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# Remodelage du muscle lisse péribronchique dans l'inflammation respiratoire chronique

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Cette thèse intitulée :

# Remodelage du muscle lisse péribronchique dans l'inflammation respiratoire chronique

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# Résumé

Le souffle chez les chevaux et l'asthme chez l'humain sont des maladies respiratoires qui partagent plusieurs caractéristiques, notamment des épisodes de bronchospasme et de détresse respiratoire dus à une inflammation pulmonaire inappropriée en réponse à une inhalation de substances antigéniques. Les manifestations cliniques incluent des efforts respiratoires augmentés, des sifflements et de la toux. Au niveau des voies respiratoires, on observe une augmentation du muscle lisse péribronchique, une fibrose sous épithéliale, une métaplasie/hyperplasie épithéliale et du mucus en quantité augmentée. L'augmentation du muscle lisse est particulièrement importante car elle n'affecte pas seulement le calibre basal des voies respiratoires, mais elle accentue l'obstruction respiratoire lors de bronchoconstriction. Ces changements sont regroupés sous le terme de « remodelage » et sont associés à un déclin accéléré de la fonction respiratoire chez les patients asthmatiques. Alors que les traitements actuels contrôlent efficacement le bronchospasme et relativement bien l'inflammation, leurs effets sur le remodelage sont mal connus. Dans le cadre de thèse, la réversibilité du remodelage musculaire péribronchique a été investiguée chez des chevaux atteints du souffle dans deux études longitudinales. Ces études, faites principalement sur du tissu pulmonaire prélevé par thoracoscopie, sont difficilement réalisables chez l'humain pour des raisons éthiques, ou chez d'autres animaux, car ceux-ci présentent rarement une inflammation de type asthmatique de façon spontanée. Les résultats démontrent que les chevaux atteints du souffle ont approximativement deux fois plus de muscle péribronchique que les chevaux sains d'âge similaire gardés dans les mêmes conditions, et que la prolifération des myocytes contribue à cette augmentation. Ils démontrent aussi qu'une stimulation antigénique relativement brève chez des chevaux atteints du souffle depuis plusieurs années n'accentue pas le remodelage, ce qui suggère que l'augmentation du muscle lisse atteint un plateau. Nous avons également montré que le remodelage du muscle lisse chez des chevaux adultes est partiellement réversible et que cette réversibilité peut être accélérée par l'administration de corticostéroïdes par inhalation.

Il semble toutefois qu'une portion du remodelage chronique est irréversible puisque les corticostéroïdes ont accéléré la diminution du muscle mais sans toutefois mener à une amélioration plus marquée au terme de l'étude qu'avec une modification environnementale stricte. La diminution de trente pourcent observée sur un an paraît modeste mais elle démontre clairement, et pour une première fois, que le remodelage du muscle lisse présent chez des chevaux adultes malades depuis plusieurs années est au moins partiellement réversible.

Mots-clés : souffle, asthme, remodelage, muscle lisse péribronchique, corticostéroïdes

# Abstract

Equine heaves and asthma in people are two respiratory diseases that share many characteristics, including episodes of bronchospasm and respiratory distress due to an inappropriate airway inflammation in response to inhaled antigens. In both diseases, the main clinical manifestations are increased breathing efforts, wheezing and coughing. Changes in the airway wall include increased airway smooth muscle, subepithelial fibrosis, epithelial changes, and increased mucus. The increase in smooth muscle is of particular importance as it not only affects baseline airway caliber, but also accentuates the effect of bronchoconstriction on airflow limitation. These structural changes are grouped under the term “remodeling” and are associated with the accelerated decline of respiratory function in asthmatics. While current treatments offer adequate control of bronchospasm and inflammation, their effects on remodeling are unknown. In this thesis, airway smooth muscle remodeling reversibility was investigated in heaves-affected horses. The longitudinal studies conducted here, mostly made on peripheral pulmonary tissue harvested under thoracoscopic guidance, can not be easily done in people for ethical reasons, or in other animal species, few of them having spontaneous asthma-like disease like horses. Results have shown that heaves-affected horses have twice as much airway smooth muscle than age-matched controls kept in the same environment, and that myocyte proliferation contributes to this increase. It was also shown that a relatively brief antigenic exposure in chronically affected horses does not further increase smooth muscle mass, which suggests that it may reach a plateau over time. It was also shown that airway smooth muscle is partially reversible and that this reversibility can be accelerated with inhaled corticosteroids. On the other hand, corticosteroids only accelerated the decrease in mass compared to strict environmental control, without affecting the total improvement observed at the end of the study, which suggests that some of this chronic remodeling is irreversible. The thirty percent decrease seems relatively modest but it is nevertheless the first

demonstration that airway smooth muscle remodeling of adult horses affected by heaves for years is at least in part reversible.

Keywords : heaves, asthma, remodeling, airway smooth muscle, corticosteroids

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# Liste des sigles et des abréviations

M1, M2, M3 : récepteurs muscariniques de type 1, 2, 3

$IP_3$ : inositol triphosphate

MLCK : myosin-light chain kinase

ATP : adénosine triphosphate

AMP : adénosine monophosphate

eNANC : système non-adrénergique non-cholinergique excitateur

iNANC : système non-adrénergique non-cholinergique inhibiteur

LT : leukotriènes

MHC : chaine lourde de la myosine

SM-A : isoforme lente de la myosine du muscle lisse

SM-B : isoforme rapide de la myosine du muscle lisse

Th1, Th2, Th17 : profil inflammatoire de type T helper 1, 2, 17

IL : interleukine

TNFα : tumor necrosis factor alpha

ADN : acide désoxyribonucléique

ARN : acide ribonucléique

MDI : metered dose inhaler

HFA : hydrofluoroalkane

DPI : inhalateurs à poudre sèche

CFC : chlorofluorocarbones

MAP kinase : mitogen-activated protein kinase

TGF : transforming growth factor

$\Delta P$ : différence de pression

$\dot{V}$ : débit

$P_{tp}$ : pression transpulmonaire

$R_L$ : résistance pulmonaire

$E_L$ : élastance pulmonaire

$V_T$ : volume courant

SSH : hybridation suppressive soustractive

PCR : polymerase chain reaction

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# Introduction

**Remodelage pulmonaire dans les maladies respiratoires inflammatoires chroniques : similitudes entre le souffle équin et l'asthme humain.** L'asthme et le souffle se caractérisent par une réponse immunitaire inappropriée à des antigènes environnementaux inhalés. Cette réponse entraîne une bronchoconstriction et une difficulté respiratoire chez les sujets susceptibles, alors que les mêmes antigènes ont peu ou pas d'effets lorsqu'inhalés par des sujets non affectés. L'asthme affecte environ 8 à 10 % des humains adultes des pays industrialisés (Canada 2005) et environ 15 % des chevaux vivants dans des climats tempérés sont atteints du souffle (Hotchkiss *et al.* 2007). Ces deux maladies ne sont pas identiques mais partagent plusieurs caractéristiques cliniques et physiopathologiques. Les épisodes d'obstruction et d'inflammation respiratoires, qui sont en grande partie réversibles, s'accompagnent de changements histologiques regroupés sous le terme de « remodelage » des voies respiratoires. Ceci inclut des changements au niveau du muscle lisse, de l'épithélium, du tissu conjonctif et des cellules à mucus. Ces éléments contribuent tous à divers degrés à l'épaississement de la paroi et au rétrécissement du calibre de la lumière des voies respiratoires. La fonction contractile du muscle lisse rend son remodelage particulièrement important, car elle décuple l'effet de son épaississement sur le calibre des voies lors de bronchospasme. Le remodelage contribue à la détérioration progressive de la fonction respiratoire observée chez les sujets asthmatiques ainsi qu'à une progression vers une réversibilité incomplète de l'obstruction au passage de l'air (Elias 2000; Kaminska *et al.* 2009; Pepe *et al.* 2005). Les effets bénéfiques de la thermoplastie (intervention qui cible spécifiquement le muscle lisse) chez les sujets asthmatiques supportent également la contribution du muscle lisse dans l'asthme (Cox *et al.* 2007). Ce remodelage est vraisemblablement secondaire à l'inflammation chronique (Munakata 2006), mais la mise en évidence de certains de ces changements chez de très jeunes patients asthmatiques suggère que les mécanismes pourraient être multiples (Tillie-Leblond *et al.* 2008). Tant chez l'homme que chez le cheval, la réversibilité du remodelage a été peu étudiée et les études portant spécifiquement sur le remodelage chronique ou celui des voies respiratoires périphériques sont encore plus rares. Ceci est en parti dû à la difficulté d'effectuer des études longitudinales impliquant de multiples biopsies pulmonaires. Les études effectuées

dans le cadre de ce travail mettent à profit les similitudes entre le souffle et l'asthme, et les conclusions qui en découlent sont bénéfiques à l'avancement des connaissances chez ces deux espèces.

## Contributions à l'avancement des connaissances

Dans l'asthme et le souffle, l'obstruction aiguë causée par le bronchospasme est en grande partie réversible mais le degré de réversibilité du remodelage structurel, et en particulier celui du muscle lisse, reste à déterminer. Il n'a pas été démontré de façon non équivoque que les corticostéroïdes, qui ont des effets sur les myocytes *in vitro,* ont également un effet sur le remodelage musculaire *in vivo*. Il est généralement accepté que les anti-inflammatoires stéroïdiens ont un effet bénéfique sur le remodelage du muscle lisse dans l'asthme, ne serait-ce qu'indirectement via un contrôle de l'inflammation, mais cette assertion reste toutefois spéculative. À de rares exceptions détaillées plus loin, l'étude de la réversibilité du remodelage musculaire dans l'inflammation pulmonaire s'effectue sur des modèles animaux chez lesquels l'inflammation et le remodelage « asthmatiques » doivent être induits, et ce sur de relativement courtes périodes. Les études réalisées dans le cadre de cette thèse ont donc pour but de combler un vide dans la recherche médicale équine et humaine sur la réversibilité du remodelage musculaire péribronchique chronique. La contribution la plus significative est d'avoir démontré que le remodelage du muscle lisse dans l'inflammation pulmonaire chronique chez des animaux adultes est partiellement réversible. La seconde contribution importante est d'avoir montré que cette réversibilité est accélérée par l'administration de corticostéroïdes, sans toutefois que ceux-ci affectent l'amélioration maximale sur une période d'un an, comparativement à un contrôle environnemental strict. Nous avons également démontré qu'il était possible d'étudier le remodelage des voies respiratoires périphériques de chevaux atteints du souffle dans des études longitudinales et non terminales, en prenant de multiples biopsies par thoracoscopie. Finalement, plusieurs observations faites dans le cadre de ces études ont également contribué à l'avancement de la médecine vétérinaire équine, notamment en comparant le traitement à long terme du souffle avec corticostéroïdes par inhalation avec une réduction de l'exposition antigénique.

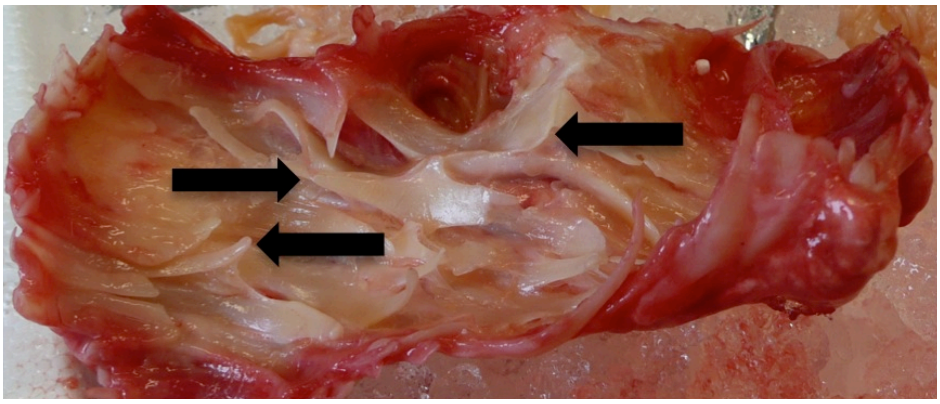
# Recension des écrits

## Description anatomique du muscle lisse péribronchique

Les poumons des mammifères sont composés d'alvéoles et d'un réseau de capillaires permettant les échanges gazeux ainsi que d'un réseau de voies respiratoires qui permettent d'acheminer l'air vers ces lieux d'échange. Dans le souffle comme dans l'asthme, l'inflammation, les anomalies fonctionnelles (bronchospasme) et les changements structurels (remodelage) se situent principalement au niveau de ces voies conductrices. Elles sont normalement composées de cellules épithéliales, d'une membrane basale, de tissu conjonctif, de muscle lisse et de cartilage, ce dernier ne s'étendant que de la trachée aux bronches distales. La paroi des voies peut également contenir des cellules inflammatoires et des vaisseaux sanguins et lymphatiques en quantité variable. Dans la portion proximale de cet arbre trachéobronchique, le muscle lisse relie les anneaux cartilagineux de la trachée et des bronches principales en s'attachant à la surface externe de ces anneaux. Ces derniers deviennent de moins en moins complets dans les bronches moyennes et distales, et prennent plutôt la forme des lames incurvées qui s'imbriquent les unes dans les autres (**Figure 1A**). À ce niveau, le muscle lisse passe rapidement de la surface externe du cartilage à une position plus centrale, à l'intérieur de celui-ci (Fixman *et al.* 2005). Le muscle est aussi présent dans les voies périphériques dépourvues de cartilage et le terme « muscle péribronchique » inclut en fait à la fois le muscle péribronchique et péribronchiolique, c'est-à-dire des voies respiratoires avec et sans cartilage, respectivement. Occasionnellement, on peut même retrouver du muscle lisse au niveau des canaux alvéolaires (Robinson 2007). Le terme anglais « airway smooth muscle » utilisé dans les articles permet d'éviter cette distinction anatomique. Ici, uniquement le terme « péribronchique » sera utilisé pour désigner le muscle péribronchique *et* péribronchiolique, afin de ne pas alourdir le texte. Le muscle péribronchique ne constitue pas des anneaux complets perpendiculaires à l'axe longitudinal des voies respiratoires et encore moins un cylindre continu, mais plutôt une structure hélicoïdale avec plusieurs faisceaux qui s'entrecroisent (**Figure 1B**). Sa contraction entraîne donc non seulement une diminution du

diamètre de la lumière des voies respiratoires mais également un certain raccourcissement. Il est à noter que dans les voies biopsiées par bronchoscopie (voir méthodologie), le muscle lisse est déjà positionné centralement au cartilage, ce qui permet d'en récolter avec les pinces endobronchiques (**Figure 11A**).

A



B

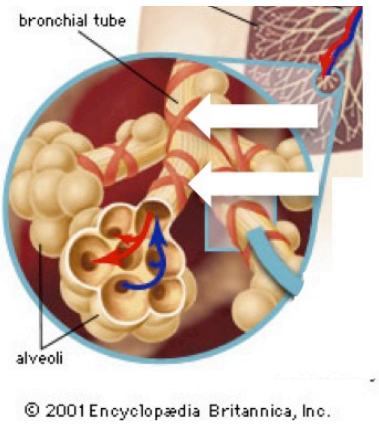



**Figure 1. Anneaux cartilagineux et muscle lisse péribronchique.**

Lames de cartilage péribronchique (A, flèches noires). Notez l'aspect des lamelles incomplètes qui s'imbriquent les unes dans les autres. Structure hélicoïdale du muscle lisse péribronchique (B, flèches blanches). Adapté de Encyclopædia Britannica, Inc.

## Rôle du muscle lisse péribronchique

Étonnamment, il n'y a pas de consensus sur le rôle du muscle péribronchique dans un poumon sain. Il est intéressant de noter que le muscle lisse péribronchique est très peu contracté de façon basale et que sa fonction physiologique semble donc être principalement liée au bronchospasme. Les fonctions proposées (résumées dans le **Tableau I**) incluent une aide à l'expulsion d'air lors de toux; un péristaltisme facilitant la croissance pulmonaire *in utero,* le déplacement de mucus vers les voies respiratoires supérieures ou le mouvement de sang et de lymphe dans les vaisseaux péribronchiques; une stabilisation des voies respiratoires; un moyen de réguler la ventilation de certaines régions afin d'optimiser le ratio ventilation/perfusion, une diminution de l'espace mort anatomique, ou la protection du poumon lors d'inhalation de particules dangereuses (James and Wenzel 2007; Mitzner 2004; Robinson 2007; Tliba and Panettieri Jr 2008). Parmi ces effets potentiellement bénéfiques, seule la réduction du calibre des voies respiratoires qui permet d'augmenter la vélocité de l'air expulsé lors de toux a été démontrée (Canning 2006). Les autres rôles potentiels sont basés sur des concepts logiques et sur certaines observations. Le rôle protecteur en cas d'inhalation de particules nocives semble à première vue intéressant mais se heurte au fait qu'une bronchoconstriction généralisée prolongée est difficilement compatible avec la vie. Même l'hypothèse d'un vestige embryonnaire, comme l'appendice ou les poils corporels chez l'humain, proposée par Mitzner (2004), reste orpheline d'une explication sur son rôle originel. Il pourrait toutefois être présent uniquement parce que l'arbre bronchique origine embryologiquement de l'intestin. De plus, les seuls processus pathologiques connus impliquant le muscle lisse péribronchique découlent d'une fonction excessive, i.e. une bronchoconstriction, et il n'y a pas d'effets néfastes connus d'une fonction diminuée (par manque de tonus, atrophie ou bronchorelaxation excessive suite à un traitement par exemple). Il n'est donc pas étonnant, dans ce contexte, que la grande majorité de la recherche dans ce domaine cherche à expliquer les processus menant au

bronchospasme prolongé dans l'asthme, tout en laissant souvent de côté la fonction physiologique du muscle lisse péribronchique.

| **Tableau I. Rôles possibles du muscle lisse péribronchique.** |
|---|
| Diminution du calibre des voies respiratoires |
| - Accélération la vélocité de l'air lors de toux<br>- Protection contre des substances inhalées toxiques<br>- Optimisation du ratio ventilation/perfusion<br>- Réduction de l'espace mort |
| Péristaltisme |
| - Croissance des voies respiratoires *in utero*<br>- Mouvement du mucus, du sang ou de la lymphe |
| Stabilisation de la paroi des voies respiratoires |

## Particularités du muscle lisse péribronchique

Une des particularités du muscle lisse réside dans sa capacité à s'adapter à son degré d'étirement et d'ainsi conserver une capacité de contraction optimale peu importe sa longueur de départ (Bai *et al.* 2004). Ce phénomène, connu sous le terme de « length-adaptation », le différencie du muscle squelettique qui possède un degré d'étirement fixe pour lequel le chevauchement des filaments d'actine et de myosine est optimal pour la génération d'une force mécanique maximale (Gordon *et al.* 1966). L'importance du phénomène d'adaptation est illustrée par la vessie urinaire, qui conserve sa capacité de contraction malgré une grande variation de distension. Pour le muscle lisse péribronchique, ce phénomène est également présent mais ne semble pas bénéfique en soi, étant donné qu'il contribue à accentuer le bronchospasme en préservant une capacité de contraction maximale même une fois le muscle partiellement contracté (Bosse *et al.* 2010). Toutefois, une particularité du muscle péribronchique est d'être soumis à des variations d'étirement oscillatoires. Comme l'adaptation à un nouveau degré d'étirement requiert une

réorganisation du cytosquelette qui passe par une phase de relaxation avant de régénérer sa capacité à produire une certaine tension, l'oscillation constante causée par la respiration permet de diminuer la réactivité du muscle péribronchique (Fredberg *et al.* 1997; Raqeeb *et al.* 2010). Il en résulte une diminution de sa capacité de contraction (Fredberg *et al.* 1999; Gunst 1983; Shen *et al.* 1997b), une augmentation du calibre des voies respirations et une diminution de la résistance au passage de l'air (Shen *et al.* 1997a; Tepper *et al.* 1995; Warner and Gunst 1992). Ces phases de relaxation sont d'autant plus marquées que les inspirations sont profondes et elles ont un effet bronchodilatateur important chez les sujets sains (Skloot *et al.* 1995).

Une autre caractéristique du muscle lisse qui le distingue du muscle squelettique est sa capacité à maintenir une contraction persistante et soutenue tout en dépensant relativement peu d'énergie. Cet état (« latch state » en anglais) semble possible grâce à une altération de la myosine, soit par une déphosphorylation de sa chaine régulatrice légère (Dillon *et al.* 1981), soit par une phosphorylation de seulement une de ses deux têtes (Tanaka *et al.* 2008). Ce phénomène est surtout important dans le muscle lisse artériel, car il permet de maintenir une pression constante, mais il est également présent dans le muscle péribronchique. Ces deux types de muscle lisse sont donc considérés « toniques », alors que le muscle lisse intestinal est plutôt « phasique ».

## Régulation de la bronchoconstriction

Le muscle lisse est l'organe effecteur principal dans le processus qui mène à l'obstruction respiratoire aiguë dans l'asthme (Lambert and Pare 1997; Macklem 1996). On estime également que la majeure partie de l'augmentation de la résistance pulmonaire dans le souffle est due à la bronchoconstriction puisque la résistance chute rapidement de 60 à 70 % avec l'administration de bronchodilatateurs (Broadstone *et al.* 1988; Derksen *et al.* 1992; Robinson *et al.* 1993). Physiologiquement, le calibre des voies respiratoires est déterminé par la balance entre les bronchoconstricteurs et les bronchorelaxants. Cette

section sur la régulation de la bronchoconstriction vise à expliquer les mécanismes qui mènent au bronchospasme et à lier celui-ci à l'inflammation.

## Innervation parasympathique cholinergique

L'innervation parasympathique cholinergique fournit le principal contrôle autonome du tonus du muscle lisse péribronchique. L'acétylcholine est libérée au niveau des terminaisons nerveuses parasympathiques qui proviennent du nerf vague, dont l'origine se situe dans le tronc cérébral (Canning and Undem 1994). Il existe trois types de récepteurs muscariniques dans l'arbre respiratoire, soit M1, M2 et M3. Ils sont situés sur les cellules musculaires, les glandes à mucus, l'endothélium vasculaire et les terminaisons nerveuses parasympathiques elles-mêmes. Au niveau musculaire, les récepteurs M2 et M3 post-synaptiques ont un effet bronchoconstricteur, alors que les récepteurs M2 pré-synaptiques ont un effet bronchorelaxant. L'action principale de l'acétylcholine est bronchoconstrictive, et ce, tant sur les voies respiratoires de large que de faible calibre, du moins chez les humains et les chevaux (Barnes 1993; LeBlanc *et al.* 1991; Mason *et al.* 1989). Cet effet bronchoconstrictif se produit grâce à la liaison de l'acétylcholine aux récepteurs M3 (Coulson and Fryer 2003). Leur activation entraîne une augmentation de l'inositol triphosphate ($IP_3$) qui se lie à des récepteurs présents sur le réticulum sarcoplasmique et mène à la libération de calcium au niveau intracellulaire. Le complexe calcium ($Ca^{2+}$) - calmoduline active une enzyme kinase, la « myosin-light chain kinase » ou MLCK. Cette enzyme permet la phosphorylation de la tête de myosine, sa liaison avec une molécule d'adénosine triphosphate (ATP), et son changement de conformation qui mène à son attachement et à son glissement avec l'actine (Somlyo *et al.* 2003). C'est ce mouvement de la tête de myosine, qui se répète plusieurs fois par seconde, et entre des milliers de filaments de myosine et d'actine à la fois, qui permet la contraction musculaire telle qu'observée macroscopiquement. Ce lien acétylcholine - récepteur M3 est à la base de la contraction musculaire; toutefois, de nombreux raffinements et particularités existent et deviennent importants dans le contexte des maladies inflammatoires obstructives. D'autres

récepteurs à l'acétylcholine sont présents sur les cellules musculaires lisses et facilitent la contraction en inhibant la relaxation induite par la stimulation des récepteurs $\beta_2$-adrénergiques. Ces récepteurs (M2) agissent en empêchant l'activation de l'adénylcyclase et des canaux potassiques calcium-dépendants (Fernandes *et al.* 1992; Kume and Kotlikoff 1991; Schramm *et al.* 1995). D'autres récepteurs M2 sont présents sur les terminaisons nerveuses pré-synaptiques et jouent au contraire un rôle visant à limiter la bronchoconstriction par un mécanisme de rétroaction négative (Coulson and Fryer 2003; Fryer and Jacoby 1998). Ces derniers récepteurs neuronaux semblent jouer un rôle dans l'asthme et certains modèles animaux (voir plus bas), incluant possiblement le souffle (Zhang *et al.* 1999). En résumé, le système nerveux autonome parasympathique a un effet principalement bronchoconstricteur, mais c'est sa portion pré-synaptique « bronchorelaxante » qui pourrait être altérée dans l'asthme. La **Figure 2** illustre l'innervation cholinergique et ses différents récepteurs.

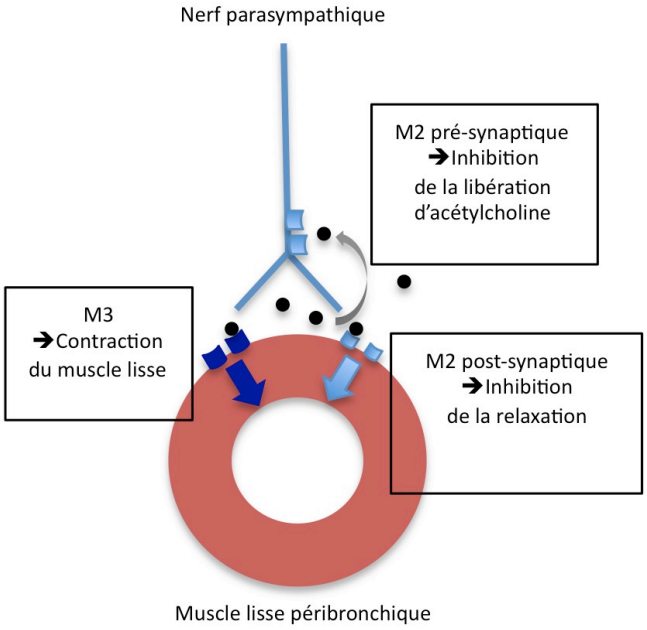



**Figure 2. Innervation parasympathique des voies respiratoires.**

•: Acétylcholine. M2 (bleu pâle) et M3 (bleu foncé): récepteurs à l'acétylcholine muscariniques de type 2 et 3. La stimulation des récepteurs M3 et M2 post-synaptiques a un effet bronchoconstricteur alors que la stimulation des récepteurs M2 pré-synaptiques a un effet bronchodilatateur, via une rétroaction négative sur la libération d'acétylcholine.

## Innervation sympathique adrénergique

Le système nerveux autonome sympathique ne dispose pas d'un réseau de fibres nerveuses élaboré dans l'arbre bronchique. Des récepteurs adrénergiques de type $\beta_2$ sont néanmoins présents sur le muscle lisse et ont pour ligands les catécholamines relâchées dans la circulation sanguine par les glandes surrénales (Barnes 1986). Chez les chevaux, les récepteurs sont surtout situés au niveau des voies respiratoires de large diamètre (Sonea *et al.* 1994). L'activation des récepteurs $\beta_2$ a un effet bronchorelaxant direct sur le muscle lisse via l'activation d'un complexe protéine $G_s$ – adénylcyclase. Il s'ensuit une accumulation

d'adénosine monophosphate (AMP) cyclique qui active la protéine kinase A et fait diminuer le calcium ($Ca^{2+}$) intracellulaire (Johnson 1998; Matera *et al.* 2002). De plus, la stimulation de récepteurs adrénergiques (incluant $\beta_2$) situés sur les terminaisons nerveuses parasympathiques inhibe la libération d'acétylcholine (Johnson 1998; Rhoden *et al.* 1988), mais il n'est pas certain que le mécanisme soit identique chez le cheval (Matera *et al.* 2002). L'effet inverse, soit une facilitation paradoxale de la libération d'acétylcholine suite à la stimulation de récepteurs $\beta_2$-adrénergiques pré-synaptiques, a même été décrit chez différentes espèces, incluant les chevaux (Belvisi *et al.* 1996; Zhang *et al.* 1995a; Zhang *et al.* 1995b). Dans des études *ex vivo* de tissus équins, il a été observé que l'effet relaxant du système sympathique adrénergique est principalement présent au niveau du muscle lisse trachéal et que la relaxation des bronches est surtout sous le contrôle du système non-adrénergique non-cholinergique (NANC) (voir paragraphe suivant) (Sonea *et al.* 1993). Ceci est toutefois partiellement contredit par l'effet bénéfique des bronchodilatateurs $\beta_2$-agonistes sur la résistance pulmonaire des chevaux atteints du souffle (Robinson *et al.* 1993). Il est possible que cette apparente contradiction indique que les récepteurs $\beta_2$-adrénergiques jouent un rôle plus important dans le bronchospasme induit par une stimulation antigénique que lorsque le muscle est stimulé par champ électrique *ex vivo*.

## Système non-adrénergique non-cholinergique (NANC)

Il existe des réponses de type neuronal qui ne passent ni par les récepteurs à l'acétylcholine, ni par les récepteurs adrénergiques et qui sont regroupées sous le terme NANC (Grundstrom *et al.* 1984). Ce système peut être excitateur (eNANC) ou inhibiteur (iNANC). L'innervation eNANC rejoint non seulement le muscle lisse, mais aussi d'autres types cellulaires comme les cellules à mucus et les vaisseaux sanguins. Les peptides agissant comme neurotransmetteurs « non traditionnels » excitateurs sont les tachykinines, ce qui inclut entre autres la substance P et les neurokinines. Les tachykinines induisent à la fois une contraction musculaire directe et facilitent la libération d'acétylcholine des terminaisons nerveuses parasympathiques (Barnes *et al.* 1991). Ce système est retrouvé

chez différentes espèces et des récepteurs à certaines tachykinines ont été mis en évidence chez les chevaux (Sonea *et al.* 1999). De son côté, la réponse inhibitrice (bronchorelaxante) du système NANC est principalement médiée par l'oxyde nitrique, et ce chez les chevaux (Yu *et al.* 1994a) comme chez les autres espèces (John *et al.* 1993).

## Bronchospasme dans l'asthme et le souffle

Les épisodes de bronchospasme dans l'asthme et le souffle peuvent s'expliquer de différentes façons. Les médiateurs de l'inflammation jouent un rôle majeur, mais le phénomène semble complexe et sera détaillé dans cette section. Le rôle du système nerveux autonome et les altérations intrinsèques du muscle lisse ont été explorés pour tenter d'expliquer certains phénomènes comme l'hyperréactivité bronchique persistante malgré un contrôle de l'inflammation chez les patients asthmatiques et les chevaux atteints du souffle asymptomatiques (Pauwels *et al.* 1988; van Erck *et al.* 2003). Le rôle de l'inflammation dans le bronchospasme, les altérations du système nerveux autonome et de ses récepteurs, ainsi qu'une possible hyperréactivité / hypercontractilité intrinsèque du muscle lisse « asthmatique » seront abordés successivement dans cette section.

### Rôle de l'inflammation

L'inflammation pulmonaire joue un rôle important dans le bronchospasme via divers mécanismes. Plusieurs substances libérées par les cellules inflammatoires activées ont un effet directement pro-contractile sur le muscle lisse (Matera *et al.* 2002). L'histamine, la sérotonine et certaines leucotriènes (LT), notamment la $LTD_4$, stimulent leurs récepteurs respectifs soit H1, 5HT et CysLT1 qui sont présents sur les cellules musculaires (Devillier *et al.* 1999; Leff 1982; Yoshida *et al.* 1999). De plus, ces substances ont un effet indirect sur le muscle en favorisant la libération d'acétylcholine au niveau des terminaisons nerveuses (Matera *et al.* 2002). Cette libération peut se faire via une stimulation des nerfs ou via un réflexe vagal, en activant les récepteurs des substances irritantes présents dans l'arbre trachéobronchique (Abela and Daniel 1994; Hey *et al.* 1992). Le **Tableau II** résume

les principaux bronchoconstricteurs impliqués dans l'asthme. La majorité de ces molécules sont impliquées dans la réponse inflammatoire. Ceci inclut les métabolites lipidiques de l'acide arachidonique (leucotriènes, prostaglandines, thromboxane), les amines (histamine, sérotonines, adénosines), et les peptides comme la bradykinine et l'endothéline. Chez les chevaux, comme chez les autres espèces, il a été démontré que l'histamine, la sérotonine et la $LTD_4$ augmentent la libération d'acétylcholine au niveau des terminaisons nerveuses (Olszewski *et al.* 1999). Le rôle de l'inflammation est tel qu'il est possible de contrôler les symptômes associés à l'asthme et au souffle uniquement avec une thérapie anti-inflammatoire, sans administrer de bronchodilatateurs ou diminuer l'exposition aux antigènes environnementaux (Jonas *et al.* 2008; Leclere *et al.* 2010b). Il est à noter que les médiateurs de l'inflammation n'affectent pas seulement le bronchospasme, mais ils ont aussi des effets sur la vascularisation, l'œdème et la sécrétion de mucus. Ce dernier effet sur les cellules à mucus est assez important, étant donné que l'accumulation de mucus joue un rôle dans l'obstruction des voies respiratoires dans le souffle et l'asthme (Gerber *et al.* 2000; Jefcoat *et al.* 2001; Kaup *et al.* 1990; Voynow and Rubin 2009).

| **Tableau II. Principaux bronchoconstricteurs dans l'asthme.** | | | |
|---|---|---|---|
| **Agoniste *** | **Récepteur *** | **Effet broncho-constricteur direct **** | **Effet neuronal **** |
| Cystéinyl leucotriènes ($LTC_4/D_4/E_4$) | $CysLT_1R$ | +++ | +/- |
| $PGF_{2\alpha}$ | FP | ++ | + |
| $PGD_2$ | $DP_2$ | ++ | + |
| Thromboxanes | TP | ++ | + |
| Histamine | $H_1$ | ++ | + |
| Endothéline-1 | $ET_A/ET_B$ | +++ | + |
| Bradykinine | $BK_1$ | + | |
| Adénosine | $A_1$ | +/- | + |
| Sérotonine | $5\text{-}HT_2/5\text{-}HT_{2c}$ | - | + |

* Adapté de (Pelaia *et al.* 2008) *et al.* Molecular mechanisms underlying airway smooth muscle contraction and proliferation: Implications for asthma. Respiratory Medicine. 2008. ** Adapté de Barnes et al. Inflammatory Mediators of Asthma: An Update. Pharmacological reviews. 1998. (Barnes *et al.* 1998). $PGF_{2\alpha}$ et $PGD_2$: prostaglandines $F_{2\alpha}$ et $D_2$.

## Altération du système nerveux autonome

Le bronchospasme dans l'asthme et le souffle est intimement lié à la présence de médiateurs inflammatoires qui participent à l'activation des récepteurs muscariniques via la libération d'acétylcholine (un effet indirect de l'inflammation sur le bronchospasme mentionné dans le paragraphe précédent) ainsi que via une diminution des mécanismes inhibiteurs (Crimi *et al.* 2001; Robinson *et al.* 1996). Il est également possible que des altérations spécifiques du système nerveux autonome soient présentes et celles-ci sont détaillées dans cette section.

### Récepteurs cholinergiques du système nerveux parasympathique

L'hyperréactivité bronchique pourrait en théorie être due à une augmentation du nombre ou de la sensibilité des récepteurs muscariniques au niveau musculaire. Cette hypothèse est intéressante car elle permet d'expliquer de façon simple l'hyperréactivité bronchique tant en présence qu'en absence d'inflammation. Il n'y a toutefois pas d'évidence que les sujets asthmatiques aient plus de récepteurs muscariniques M3, ni que ceux-ci aient un seuil d'excitation plus bas que ceux des sujets non asthmatiques (résumé par (Coulson and Fryer 2003)). Des études ont même montré l'inverse, à savoir que le muscle lisse péribronchique des patients asthmatiques était moins sensible *ex vivo* à des agonistes cholinergiques (Goldie *et al.* 1986), ce qui laisse supposer que l'hyperréactivité bronchique est en grande partie liée au milieu inflammatoire retrouvé *in vivo*. Il semble toutefois que d'autres types de récepteurs muscariniques pourraient être altérés. Tel que mentionné dans la section « Régulation de la bronchoconstriction », les récepteurs M2 post-synaptiques facilitent la contraction en diminuant la stimulation des récepteurs $\beta_2$, et une altération de cette fonction est présente chez certains modèles d'hyperréactivité bronchique (Fryer and Jacoby 1998). Une dysfonction des récepteurs M2 pré-synaptiques (« bronchorelaxants ») a également été observée dans l'asthme et chez des modèles animaux (Ayala and Ahmed 1989; Larsen *et al.* 1994; Minette *et al.* 1989; Mitchell *et al.* 1987; ten Berge *et al.* 1996). Certains considèrent même que la dysfonction de ces récepteurs est la principale cause de

l'augmentation du tonus parasympathique observé dans l'asthme (Coulson and Fryer 2003), même s'il n'est toujours pas clair s'il s'agit d'une anomalie intrinsèque ou d'une altération secondaire à la présence de cytokines et autres médiateurs inflammatoires (Fryer *et al.* 1999; Gambone *et al.* 1994). De plus, même si le tonus parasympathique est effectivement augmenté dans l'asthme, il ne semble pas que ce soit la cause principale de la maladie (Goyal *et al.* 2010). De façon similaire, il n'y a pas d'évidence d'une réponse exagérée à l'acétylcholine au niveau du muscle lisse des chevaux atteints du souffle (Broadstone *et al.* 1991; LeBlanc *et al.* 1991; Yu *et al.* 1994b) ni, plus spécifiquement, de la fonction et de l'expression de ses récepteurs (Abraham *et al.* 2007). Il existe possiblement une dysfonction de la rétroaction négative via une diminution des récepteurs M2 pré-synaptiques (Zhang *et al.* 1999). Cette dysfonction n'a toutefois pas pu être mise en évidence dans une autre étude du même groupe (Wang *et al.* 1995) ni par d'autres auteurs (Abraham *et al.* 2007), et reste donc spéculative.

**Récepteurs $\beta_2$-adrénergiques du système nerveux sympathique**

Une malfonction des récepteurs $\beta_2$-adrénergiques pourrait aussi mener à une hypersensibilité du muscle lisse. Cette théorie, proposée dès 1968 par Szentivanyi dans un article largement cité par la suite, postule qu'une anomalie congénitale ou acquise du complexe récepteurs $\beta_2$-adrénergiques/AMP cyclique résulterait en une hyperréactivité bronchique, spécifique et non spécifique, telle qu'observée dans l'asthme (Szentivanyl 1968). Des études *in vitro* ont montré que la capacité de plusieurs agonistes $\beta_2$-adrénergiques à contrecarrer une contraction musculaire induite par l'acétylcholine est réduite chez les sujets asthmatiques (Bai 1991; Goldie *et al.* 1986). Les études sur la quantification des récepteurs $\beta_2$-adrénergiques sont toutefois contradictoires; quelques unes ont montré une diminution des récepteurs (Szentivanyi *et al.* 1979), alors que plusieurs ont montré au contraire une augmentation de leur nombre ou de leur ARN messager (Bai *et al.* 1992; Bai *et al.* 1993; Spina *et al.* 1989), et ceci, même dans les études ayant montré une diminution de l'activité des agonistes $\beta_2$-adrénergiques (Bai 1991; Bai *et al.* 1992). Finalement, quelques études n'ont simplement pas permis de démontrer de différence de

densité des récepteurs dans le poumon ou dans le muscle péribronchique de sujets asthmatiques (Haddad *et al.* 1996; Sharma and Jeffery 1990). Il semble donc qu'il pourrait y avoir une anomalie fonctionnelle des récepteurs $\beta_2$ dans l'asthme, mais pas nécessairement de modification de leur nombre. Chez des modèles expérimentaux d'asthme, une altération de la sensibilité des récepteurs $\beta_2$-adrénergiques à l'acétylcholine a été montrée (Hakonarson *et al.* 1995; Rubinfeld *et al.* 1982; Taki *et al.* 1986; Wills-Karp and Gilmour 1993). Chez les chevaux atteints du souffle, la densité des récepteurs $\beta_2$-adrénergiques est apparemment diminuée et l'affinité du récepteur pour la protéine $G_s$ est également anormale (Abraham *et al.* 2006). La protéine $G_s$, en activant l'adénylcyclase qui synthétise de l'AMP cyclique à partir de l'ATP, induit la relaxation musculaire. Bien que les altérations fonctionnelles des récepteurs $\beta_2$-adrénergiques semblent présentes chez plusieurs espèces, quelques auteurs ont émis des doutes sur la capacité de cette anomalie d'expliquer l'hyperréactivité bronchique dans son ensemble et y voient, au mieux, un facteur contribuant à l'hyperréactivité ou une conséquence de l'inflammation chronique (Bai 1992). En d'autres termes, la fonction bronchorelaxante $\beta_2$-adrénergique pourrait être altérée à cause du microenvironnement inflammatoire et non être le problème primaire dans l'asthme, comme ce qui est proposé pour les altérations du tonus parasympathique. Pour ajouter à la confusion sur le sujet, les β-bloquants, surtout utilisés dans les maladies cardiaques, ont longtemps été potentiellement contre-indiqués chez les patients asthmatiques (Schwartz *et al.* 1980; Vatrella *et al.* 2001) alors que des données récentes indiquent qu'ils pourraient être bénéfiques dans certaines circonstances, possiblement en augmentant la concentration de $\beta_2$-récepteurs (Morales *et al.* 2011).

**Système non-adrénergique non-cholinergique**

Comme les autres altérations du système nerveux autonome, les altérations non-adrénergiques non-cholinergiques (NANC) observées dans l'asthme sont possiblement secondaires à l'inflammation pulmonaire. Certaines tachykinines du système NANC excitateur (eNANC) sont augmentées dans des modèles animaux, mais leur rôle est moins bien défini chez les humains (Kraneveld *et al.* 2000). L'oxyde nitrique, qui fait partie du

NANC inhibiteur (iNANC) et qui a un effet bronchorelaxant, est paradoxalement également augmenté chez les sujets asthmatiques (Wechsler *et al.* 2000), possiblement en lien avec ses autres fonctions, notamment inflammatoires (Goyal *et al.* 2010). Finalement, bien que les systèmes NANC excitateur et inhibiteur soient présents chez les chevaux, leur contribution au bronchospasme dans le souffle reste encore à définir (Matera *et al.* 2002).

## Anomalies intrinsèques du muscle lisse

Une autre façon d'expliquer la contraction excessive du muscle péribronchique serait la présence de propriétés contractiles altérées chez les sujets asthmatiques et les chevaux atteints du souffle. Présentement, il n'y a pas de consensus sur la présence d'une contractilité anormale chez les myocytes péribronchiques de sujets asthmatiques ou dans les modèles animaux d'asthme (An *et al.* 2007). Les principales hypothèses portant sur les anomalies musculaires intrinsèques et les données les supportant sont détaillées ici.

### Augmentation de la force de contraction

Quelques études *in vitro* et *ex vivo* ont permis d'observer une augmentation de la force de contraction du muscle lisse (Florio *et al.* 1996; Gil *et al.* 2006). Cependant cette observation ne fait pas l'unanimité et certains ont observé une contractilité similaire, tout en notant une vélocité élevée (voir paragraphe suivant) (Jiang *et al.* 1992; Mitchell *et al.* 1994).

### Augmentation de la vitesse de contraction

La vitesse de contraction influence le degré de bronchoconstriction. Ce phénomène est moins intuitif qu'une simple augmentation de la force de contraction mais comme le muscle lisse péribronchique est soumis à des oscillations dues à la respiration (voir « Particularités du muscle lisse péribronchique »), une contraction plus rapide se traduit par une bronchoconstriction plus marquée. En effet, si les fibres musculaires parviennent à se contracter rapidement entre deux respirations, la période de relaxation suivante peut être insuffisante pour permettre un retour à leur longueur initiale, ce qui fait que le muscle

pourra se contracter encore davantage au cycle suivant. Ce phénomène est accentué par l'adaptation à l'élongation ou « length adaptation » décrite précédemment et qui permet au muscle lisse de maintenir une contraction maximale à des niveaux d'élongation variables. Entre autres observations, une augmentation de la vitesse de contraction a été mise en évidence chez le rat Fisher, un race qui présente une hyperréactivité bronchique innée (Blanc *et al.* 2003; Leguillette *et al.* 2009; Tao *et al.* 1999; Wang *et al.* 1997). Tao et collègues ont aussi démontré que la contraction à la fois plus rapide et de plus grande amplitude chez cette lignée de rat est associée à une augmentation exagérée de la concentration intracellulaire de calcium lors de stimulation par un agoniste contractile (Tao *et al.* 1999). Toujours chez le rat Fisher, la contraction plus rapide pourrait aussi s'expliquer par une plus grande concentration de l'isoforme rapide de la myosine (SM-B) et par une augmentation du degré de phosphorylation de sa chaine régulatrice (Gil *et al.* 2006). Chez les sujets asthmatiques, certaines études (Leguillette *et al.* 2009), mais pas toutes (Ma *et al.* 2011), ont permis d'observer une augmentation de la proportion de l'isoforme rapide de la myosine dans des biopsies endobronchiques de patients asthmatiques. D'autres ont mis en évidence une plus grande expression de MLCK (myosin light chain kinase), ce qui peut aussi expliquer une augmentation de la vitesse de contraction (Leguillette *et al.* 2009; Stephens *et al.* 2003).

**Résistance à l'effet relaxant de l'oscillation**

Tel que décrit précédemment, la respiration normale a un effet bronchodilatateur important en situation physiologique (Gump *et al.* 2001) et il est possible que l'hyperréactivité bronchique soit due à une résistance, intrinsèque ou acquise, à la relaxation. Selon cette hypothèse, le muscle lisse des sujets asthmatiques serait non pas hypercontractile mais plutôt résistant à la relaxation. L'effet bronchorelaxant négligeable des inspirations profondes chez les patients asthmatiques est connu depuis près de deux siècles (Fish *et al.* 1981; Salter 1990; Wheatley *et al.* 1989), mais le phénomène reste mal compris au niveau cellulaire. Il est possible que les myocytes des patients asthmatiques aient des filaments d'actine plus longs, ce qui rendrait les liaisons plus stables et donc moins facile à

désorganiser (Solway *et al.* 2003). D'autres ont émis l'hypothèse que la résistance à la relaxation est plutôt liée au remodelage. Oliver et collègues ont en effet montré que la seule augmentation de l'épaisseur du muscle lisse est suffisante pour engendrer une résistance à la bronchorelaxation, indépendamment de modifications fonctionnelles ou de l'épaississement des autres composantes de la paroi des voies respiratoires (Oliver *et al.* 2007). Ce phénomène n'a pas été étudié chez les chevaux atteints du souffle mais les inspirations profondes étant présentes chez la plupart des mammifères, incluant le cheval, il est probable que le remodelage ait un effet similaire (Robinson 2007).

En conclusion de cette section sur le bronchospasme dans l'asthme et le souffle, il semble qu'il n'y ait pas d'anomalies intrinsèques de l'innervation sympathique ou parasympathique, mais plutôt des changements secondaires à l'inflammation pulmonaire, et que cette dernière joue un rôle prépondérant dans le bronchospasme. Il semble également que même s'il y a des données convaincantes sur les propriétés contractiles anormales des myocytes provenant de rongeurs avec une hyperréactivité bronchique innée, il n'y a pas de consensus sur une contractilité accrue des myocytes des sujets asthmatiques. Enfin, la résistance à la relaxation induite par les inspirations profondes est un phénomène bien connu mais encore mal expliqué. Une des hypothèses est liée à l'augmentation de la masse muscle lisse, un des aspects du remodelage qui est décrit dans la section suivante.

## Remodelage du muscle péribronchique dans l'asthme et le souffle

Les sections précédentes décrivaient principalement l'innervation et les propriétés contractiles du muscle péribronchique, sans aborder directement l'augmentation de la masse observée dans l'asthme et le souffle. Pour plusieurs auteurs, le terme « remodelage » englobe à la fois l'augmentation de la quantité de muscle lisse et les changements phénotypiques des myocytes. Ces changements seront donc aussi abordés dans cette section.

L'importance du remodelage pulmonaire dans l'asthme vient principalement de son effet sur la fonction respiratoire. Le remodelage pulmonaire des patients asthmatiques peut à la fois exacerber le degré d'obstruction lors de bronchospasme aigu et contribuer au déclin de la fonction respiratoire en dehors des épisodes de bronchospasme (Lange *et al.* 1998; O'Byrne *et al.* 2009). De façon générale, le remodelage semble être une conséquence des mécanismes inflammatoires qui aident normalement à combattre les agents pathogènes et contribuent à la réparation tissulaire, mais qui deviennent dommageables lorsque la réponse inflammatoire est exagérée. Ces cycles de dommages et réparations répétés entraînent plusieurs changements structuraux plus ou moins réversibles, notamment de l'hyperplasie épithéliale et des cellules à mucus, de la fibrose sous épithéliale, de l'angiogénèse, en plus de l'augmentation de la masse du muscle lisse (**Figure 3**). Ces changements contribuent à un épaississement de la paroi des voies respiratoires qui joue un rôle dans l'hyperréactivité bronchique et le déclin de la fonction respiratoire, et devraient être pris en compte dans les objectifs de traitement de l'asthme (Bai 2010; Lange *et al.* 1998).

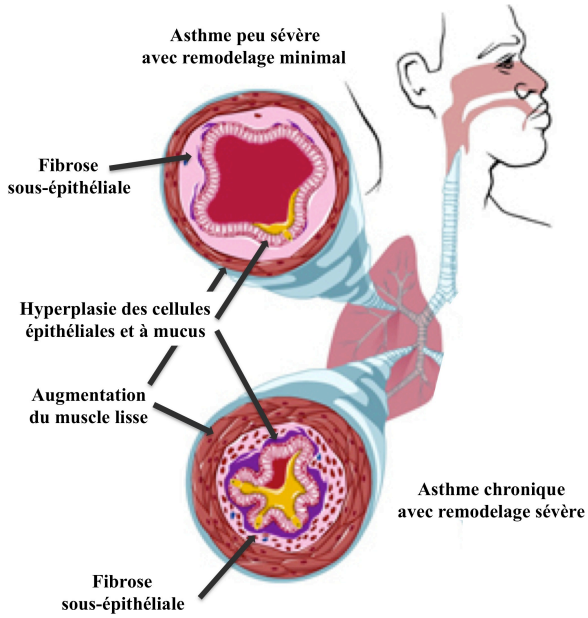



**Figure 3. Remodelage des voies respiratoires dans l'asthme.**

Le remodelage des voies respiratoires dans l'asthme inclut des changements au niveau de l'épithélium, une hyperplasie des cellules et des glandes à mucus, de la fibrose sous épithéliale, de l'angiogénèse (non illustrée) et une augmentation de la masse du muscle lisse. Adapté d'une image du domaine public : National Institute of Allergy and Infectious Diseases (NIAID).

## Remodelage du muscle lisse dans l'asthme

L'augmentation du muscle lisse contribue de deux façons à la diminution de la lumière des voies respiratoires. Premièrement, son augmentation de volume participe passivement au rétrécissement de la lumière, comme le fait l'épaississement des autres composantes de la paroi. Toutefois, à cause de sa fonction contractile, l'épaississement du muscle accentue aussi le rétrécissement de la lumière lors de bronchoconstriction, et ce de façon exponentielle (James *et al.* 1989; Lambert *et al.* 1993). Le remodelage influence donc la fonction pulmonaire basale ainsi que l'hyperréactivité bronchique (An *et al.* 2007; Lambert

*et al.* 1993). L'augmentation de la masse du muscle lisse est considérée comme une des caractéristiques histologiques prédominantes de la maladie, étant présente chez les sujets asthmatiques qu'ils soient légers, modérés ou sévères (Ebina *et al.* 1993; Hirst *et al.* 2004; James and Carroll 2000; James *et al.* 1989; Woodruff *et al.* 2004). Il faut noter qu'une augmentation de la masse de muscle lisse entraîne un bronchospasme plus sévère, et ce peu importe que chaque myocyte maintienne une contractilité similaire ou que la contractilité soit augmentée (masse et force totale augmentées, contractilité similaire versus masse, force totale et contractilité augmentées) (Lambert *et al.* 1993; Macklem 1996; Moreno *et al.* 1986). Les conséquences physiologiques de l'augmentation du muscle lisse, la contribution de l'hyperplasie, de l'hypertrophie et éventuellement d'autres facteurs (résumé par (Hirst *et al.* 2004)), ainsi que le rôle des corticostéroïdes dans la modulation du remodelage ne sont pas complètement compris à ce jour (James and Wenzel 2007; Panettieri *et al.* 2008b).

**Augmentation de la masse de muscle lisse**

Depuis le début du XX$^{e}$ siècle, de nombreuses études ont démontré une augmentation de muscle lisse dans l'asthme (Huber and Koessler 1922). Selon le type d'étude, l'origine des échantillons et la méthode de quantification, la masse de muscle lisse est estimée être augmentée de 50 à 400% dans les cas d'asthme fatal et de 25 à 50 % chez les patients asthmatiques moins sévèrement atteints (Bai *et al.* 2000; James 1997). Plusieurs études portant sur des tissus prélevés sur des patients asthmatiques décédés des suites de leur maladie (asthme fatal) ou d'autres causes (asthme non fatal) (Bai *et al.* 2000; Carroll *et al.* 1993; Dunnill *et al.* 1969; Ebina *et al.* 1993; Heard and Hossain 1971; James *et al.* 2009; Kuwano *et al.* 1993; Saetta *et al.* 1991) ont été publiées au cours des dernières décennies et supportent l'augmentation du muscle lisse dans les voies respiratoires centrales et périphériques. La quantification du muscle dans les tissus prélevés par biopsies endobronchiques de sujets atteints d'asthme léger à sévère va dans le même sens (Benayoun *et al.* 2003; Kaminska *et al.* 2009; Pepe *et al.* 2005; Woodruff *et al.* 2004). Même si l'amplitude de l'augmentation du muscle lisse est variable, il n'en reste pas moins qu'une augmentation a été observée chez tous les types de sujets asthmatiques, incluant les

enfants (Jenkins *et al.* 2003; Regamey *et al.* 2008; Tillie-Leblond *et al.* 2008), et que très peu d'études montrent l'inverse (Thomson *et al.* 1996). De plus, même si certaines données semblent lier la durée de la maladie et le degré d'augmentation du muscle lisse (Bai *et al.* 2000) une large étude multicentrique plus récente associe le remodelage musculaire à la sévérité de la maladie et non à sa durée (James *et al.* 2009).

**Mécanismes pouvant expliquer l'augmentation de masse musculaire**

Si l'augmentation du muscle lisse semble faire consensus, il n'en va pas de même pour les mécanismes qui mènent à ce remodelage. Les hypothèses principales sont que l'augmentation de masse se fait via l'hyperplasie ou l'hypertrophie des myocytes. Une augmentation de la matrice extracellulaire pourrait également faire augmenter la masse totale (James and Wenzel 2007). Enfin, on ne peut pas complètement exclure que les patients asthmatiques aient plus de muscle lisse dès la naissance, avant l'apparition de signes cliniques. Cette dernière hypothèse va toutefois à l'encontre de la théorie voulant que le remodelage soit une conséquence de l'inflammation chronique. Les principales hypothèses pouvant expliquer l'augmentation de la masse de muscle lisse dans l'asthme sont présentées dans la **Figure 4** et détaillées dans les paragraphes suivants.

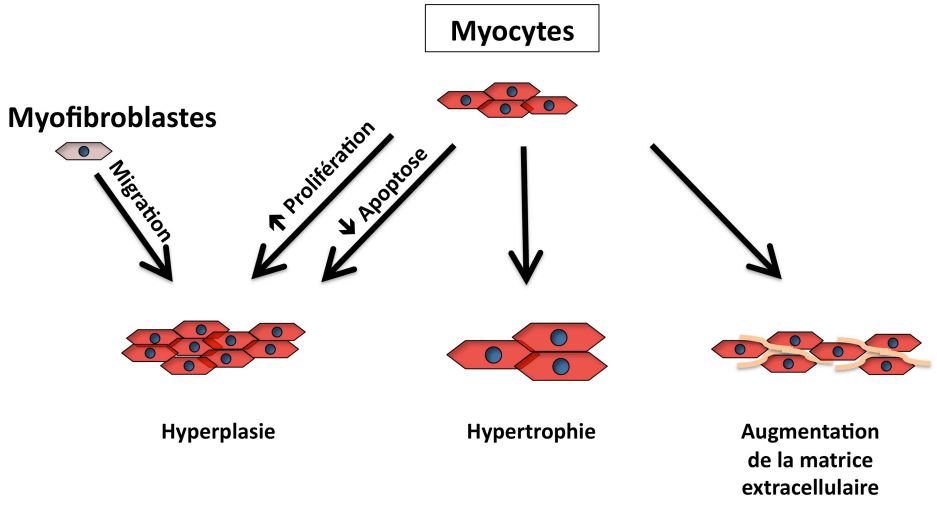



**Figure 4. Mécanismes pouvant contribuer à l'augmentation du muscle lisse.**

L'augmentation de la masse de muscle lisse peut s'expliquer soit par une augmentation du nombre de myocytes (hyperplasie), de la taille des myocytes (hypertrophie), ou de la matrice extracellulaire entre les myocytes. L'hyperplasie peut être secondaire à une augmentation de la prolifération des myocytes ou une diminution de leur apoptose, ou encore être due à la migration et la transformation de myofibroblastes en myocytes.

*Hyperplasie et hypertrophie musculaire*

Dans l'hyperplasie musculaire, l'augmentation de la masse est due à une augmentation du nombre de myocytes alors que dans l'hypertrophie, l'augmentation de masse est due à une augmentation de la taille des myocytes. L'hyperplasie du muscle lisse a été décrite chez les sujets asthmatiques (Heard and Hossain 1971; Woodruff *et al.* 2004). Il y a aussi des évidences que l'hyperplasie et l'hypertrophie contribuent toutes deux à l'augmentation de muscle lisse chez les mêmes individus (Regamey *et al.* 2008), possiblement en proportions différentes selon la taille des voies respiratoires et la distribution du remodelage (Ebina *et*

*al.* 1993). Certaines études ont toutefois mis en évidence uniquement de l'hyperplasie (sans hypertrophie) (Woodruff *et al.* 2004), alors que seule de l'hypertrophie a été observée dans d'autres études (Benayoun *et al.* 2003). La démonstration que l'hyperplasie contribue de façon significative au remodelage se bute à la difficulté de mettre en évidence ses mécanismes habituels, à savoir une augmentation de la prolifération des myocytes, ou une diminution de leur apoptose. Une prolifération exagérée est principalement supportée par des études *in vitro*. En effet, de nombreux médiateurs de l'inflammation et des facteurs de croissances retrouvés dans l'asthme ont des effets mitogènes sur les cellules musculaires (résumé par (Hirst *et al.* 2004)). Il a notamment été démontré que le liquide de lavage bronchoalvéolaire des sujets asthmatiques induit la prolifération des cellules musculaires lisses en culture (Naureckas *et al.* 1999), et que les myocytes des sujets asthmatiques sont plus sensibles aux substances mitogènes que les myocytes de sujets sains (Johnson *et al.* 2004b; Johnson *et al.* 2001). Par contre, c'est seulement récemment qu'une augmentation de marqueurs de prolifération a été observée *in situ* dans des biopsies de sujets asthmatiques (Hassan *et al.* 2010), alors que des études précédentes n'avaient pas pu mettre en évidence ce phénomène (Bamford *et al.* 2002; Benayoun *et al.* 2003; Ward *et al.* 2008). Une diminution de l'apoptose n'a pas non plus été observée chez les patients asthmatiques (Hirst *et al.* 2004). Une autre hypothèse pour expliquer une augmentation des cellules musculaires a été émise récemment, soit la migration de myocytes ou de myofibroblastes qui viendraient se joindre au muscle en place, d'une façon similaire à ce qui est observé dans le remodelage musculaire vasculaire (résumé par (Gerthoffer 2008)) (Daniel and Sedding 2011). De nombreuses substances comme des facteurs de croissance et des médiateurs de l'inflammation peuvent induire la migration des myocytes *in vitro* mais il reste à déterminer si ce phénomène a non seulement lieu *in vivo*, mais encore s'il contribue significativement à l'augmentation de la masse de muscle lisse (Hirst *et al.* 2004).

L'hypertrophie quand à elle peut être induite *in vitro* en privant les myocytes en culture de sérum (Bentley and Hershenson 2008). L'ajout de TGF (transforming growth

factor)-β au milieu favorise également l'hypertrophie des cellules musculaires lisses (Bentley and Hershenson 2008). Les cellules ainsi stimulées non seulement augmentent de taille, mais expriment plus de protéines contractiles et répondent de façon plus marquée aux agonistes contractiles (Goldsmith *et al.* 2006). La cardiotrophine et l'endothéline ont des effets similaires, sans toutefois se traduire par une augmentation de la contractilité dans le cas de la cardiotrophine (McWhinnie *et al.* 2007; Zhou *et al.* 2003), ce qui laisse penser que non seulement l'augmentation de masse ne se traduit pas toujours par une augmentation de contractilité au niveau cellulaire. Les profiles prolifératifs et contractiles des myocytes sont détaillés plus loin, sous « Changements phénotypiques du muscle lisse dans le remodelage ».

*Augmentation de la matrice extracellulaire*

La matrice extracellulaire qui entoure les myocytes peut aussi contribuer à l'augmentation de la masse totale (Bai *et al.* 2000). Cette matrice comprend des protéines comme l'élastine, la fibronectine et différents types de collagène (résumé par (Bara *et al.* 2010). Une augmentation de certaines glycoprotéines au sein de la masse de muscle lisse a été observée dans des biopsies endobronchiques de sujets atteints d'asthme modéré (Pini *et al.* 2007). Cette augmentation n'était cependant pas significative chez les patients asthmatiques sévères de la même étude, ce qui laisse penser que ce phénomène est peut-être transitoire. Son rôle est toutefois possiblement plus important que de simplement contribuer au remodelage étant donné les effets que ces protéines peuvent avoir sur le muscle (Bosse *et al.* 2008; Johnson *et al.* 2004b).

**Changements phénotypiques du muscle lisse dans le remodelage**

Les cellules musculaires mises en culture maintiennent généralement difficilement leurs propriétés contractiles et la prolifération des cellules s'accompagne d'une transition vers une augmentation de la synthèse et production de matrice extra cellulaire, des cytokines, chimiokines, des molécules d'adhésions et facteurs de croissance (Howarth *et al.* 2004). Cet aspect synthétique peut paraître quelque peu incongru de la part d'une cellule dont la

fonction première est de se contracter. Ces propriétés ont d'abord été observées *in vitro* sur des myocytes en culture. Cette transition d'un phénotype contractile à un phénotype synthétique / prolifératif s'accompagne d'une perte des protéines servant à la contraction (Hirota *et al.* 2009). À partir de ces observations faites *in vitro*, il a été suggéré que ce phénomène existait aussi *in vivo* et que les cellules musculaires devenaient plus sécrétoires lorsque le remodelage se mettait en place. Ceci est a été illustré dans un modèle d'asthme allergique chez le rat. Dans ce modèle, une stimulation antigénique induit rapidement une hyperplasie musculaire de courte durée qui s'accompagne d'une diminution de la contractilité et de l'expression de protéines associées à la contraction (myosine et MLCK) (Labonte *et al.* 2009). Dans une autre étude avec un modèle similaire mais plus chronique, une augmentation transitoire de masse musculaire et une diminution des protéines contractiles ont également été observées dans un premier temps, mais ceci a été suivi par une augmentation de la contractilité un mois plus tard, alors que la masse était revenue à son niveau basal (Moir *et al.* 2003). Ces changements sont donc apparemment dynamiques et transitoires, et il n'est toujours pas clair si cette dualité existe dans l'asthme chronique chez l'humain, ni même s'il existe vraiment un changement de phénotype (Halayko *et al.* 1996; Hirst *et al.* 2000b) ou si les phénotypes de myocytes cohabitent chez un même individu (Zuyderduyn *et al.* 2008). Pour ce qui est du profil contractile, il est souvent, mais pas toujours, associé à l'hypertrophie. De nombreuses protéines sont associées à la fonction contractile du muscle lisse (Halayko *et al.* 1996; Hirst *et al.* 2000a). Ces protéines sont souvent exprimées en plus grande quantité par les myocytes dit « contractiles » que par les myocytes montrant un profil synthétique / prolifératif, mais elles ne sont pas des marqueurs spécifiques en soit. Il existe également différentes isoformes de la myosine qui ont des vitesses de contraction différentes, selon qu'un court insert soit présent (isoforme B, rapide) ou non (isoforme A, lente) (Babu *et al.* 2004). La proportion d'isoforme B, habituellement associée au muscle lisse phasique, pourrait jouer un rôle dans le remodelage musculaire et la bronchoconstriction dans l'asthme (Leguillette *et al.* 2005). La **Figure 5** résume les principales caractéristiques des différents phénotypes décrits dans la littérature.

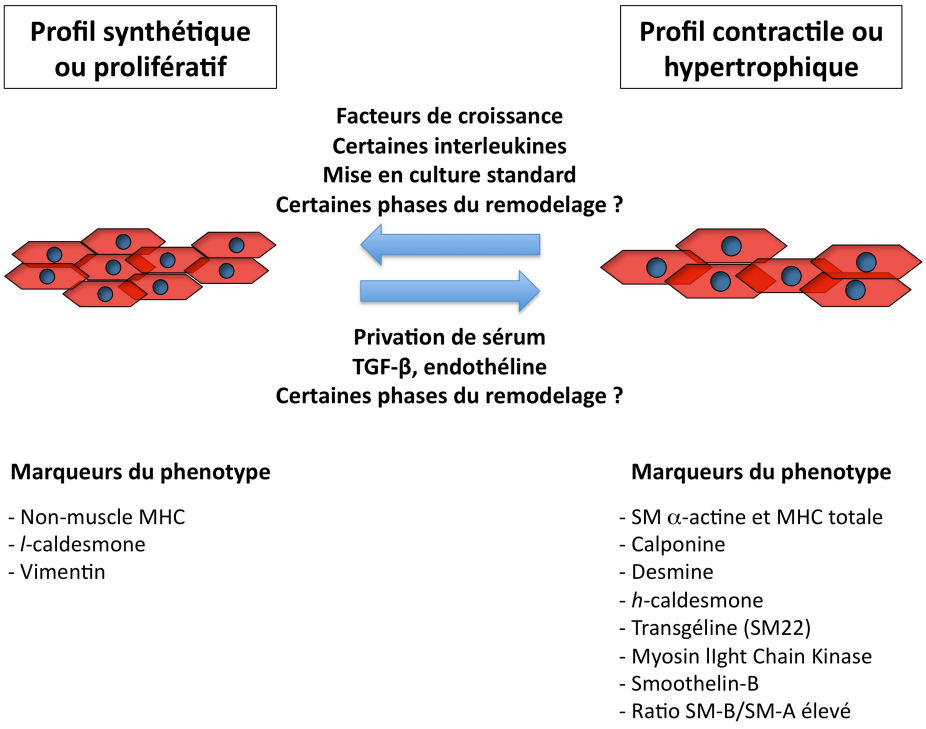



**Figure 5. Profils prolifératif et contractile des myocytes.**

TGF-β : Transforming growth factor beta. MHC : chaine lourde de la myosine, SM : muscle lisse, SM-B/SM-A : isoformes rapide (B) et lente (A) de la myosine du muscle lisse.

## Remodelage du muscle lisse dans le souffle

Avant d'entamer la description du remodelage musculaire dans le souffle, ses ressemblances avec l'asthme et sa pertinence comme modèle animal doivent être décrites de façon plus détaillée. Tel que mentionné précédemment, plusieurs résultats des travaux présentés ici sont pertinents pour l'amélioration de la santé équine, mais certains aspects

ont été investigués spécifiquement parce que l'étude du souffle peut permettre de faire avancer les connaissances dans l'asthme.

**Anatomie pulmonaire comparée des humains et des équins**

L'anatomie pulmonaire des équins partage des similitudes avec celle des humains, notamment en ce qui a trait à la circulation bronchique, la lobulation partielle, les bronchioles respiratoires faiblement développées et la présence d'anastomoses entre les circulations bronchique et pulmonaire (McLaughlin *et al.* 1961). Dans une étude d'anatomie pulmonaire comparée, McLaughlin et collègues regroupent les vaches, les moutons et les porcs dans une catégorie, les chiens, les chats et les singes dans une seconde, et finalement les chevaux et les humains dans une troisième. Malgré plusieurs similitudes, le cheval n'a pas de lobes distincts, sauf le lobe accessoire droit (Robinson and Furlow 2007), alors que chez l'humain, des lobes sont présents, bien que les fissures interlobaires soient fréquemment incomplètes (Satoh *et al.* 1996). Des septa fibreux divisent le poumon en lobules et la ventilation collatérale entre ces lobules est limitée. Chez les chevaux comme chez les humains, et contrairement aux ruminants et aux porcs, cette lobulation est incomplète (McLaughlin *et al.* 1961). Les bronches et les artères sont parallèles jusqu'en périphérie du poumon et sont reliées par du tissu conjonctif lâche qui contient également des vaisseaux lymphatiques et des nerfs. Les veines sont moins intimement associées au trajet des bronches (Robinson and Furlow 2007). La circulation bronchique est remarquablement semblable chez les chevaux et les humains (Magno 1990). Elle reçoit environ 2 pourcent du débit cardiaque et apporte éléments nutritifs et oxygène aux bronches, aux parois des larges vaisseaux et à la plèvre. Elle rejoint en partie la circulation veineuse systémique via la veine azygos mais une portion rejoint la circulation pulmonaire veineuse, créant un shunt physiologique droite - gauche. Les voies respiratoires sont composées de plusieurs générations de bronches, puis de bronchioles dépourvues de cartilage, et ensuite de canaux alvéolaires. L'arbre bronchique des équins a un type d'embranchement dit monopodial, c'est - à - dire que des bronches secondaires quittent la

bronche principale à différents sites et angles (Smith *et al.* 1994), alors que chez l'humain, les bronches se divisent de façon dichotomique, soit en deux bronches de tailles similaires, quoique souvent asymétriques (Horsfield *et al.* 1971). Chez les chevaux adultes, le diamètre interne de la trachée est en moyenne de 26 mm (dorso - ventral) par 46 mm (latéro - latéral) (Widdicombe and Pecson 2002). Smith et collègues ont pu répertorier et explorer par endoscopie jusqu'à 18 embranchements entre la carina et 35 cm caudalement à celle-ci (Smith *et al.* 1994). Les voies respiratoires avec des parois cartilagineuses sont considérées comme des bronches, se qui correspond habituellement à un diamètre d'environ 2 mm. Chez les équins, les bronchioles sont donc des voies non cartilagineuses qui font habituellement moins de 2 mm de diamètre (Bartner *et al.* 2006; Robinson and Furlow 2007), soit environ 6.5 mm de circonférence, alors qu'elles sont plutôt de moins de 1 mm chez l'humain (Kuhn 2005). L'épithélium des bronchioles est composé d'une couche de cellules cuboïdales qui incluent des cellules ciliées, des cellules de Clara (Plopper *et al.* 1980) et, en absence d'inflammation, très peu de cellules caliciformes ou à gobelet (Kaup *et al.* 1990). Les alvéoles sont regroupées dans des sacs alvéolaires, mais certaines prennent directement naissance au niveau des canaux alvéolaires ou des bronchioles respiratoires. Ces dernières sont peu ou pas présentes chez les chevaux (Tyler *et al.* 1971) et apparemment faiblement développées chez l'humain (McLaughlin *et al.* 1961). Les plèvres pariétale et viscérale sont considérées relativement épaisses chez ces deux espèces. Une particularité des plèvres chez les équins est la présence de fenestrations dans le médiastin caudal. Ces fenestrations sont facilement obstruées lors d'inflammation. Ceci a probablement peu d'impact sur l'étude du remodelage des voies respiratoires mais explique le plus grand nombre de pneumothorax bilatéraux lors de thoracoscopie chez les chevaux sains dans l'étude I.

**Le souffle comme modèle d'asthme chronique**

Notre compréhension de l'asthme a grandement progressé dans les dernières décennies grâce à l'utilisation de modèles animaux, notamment ceux qui ont été développés pour répondre à des questions spécifiques. L'utilisation de petits rongeurs comme animaux de

laboratoire a plusieurs avantages, dont leur relativement faible coût, la grande disponibilité d'outils moléculaires et immunologiques, ainsi qu'une homogénéité génétique qui facilite certaines études (Bice *et al.* 2000). Toutefois, les souris ont des différences anatomiques pulmonaires importantes avec les humains, comme une faible quantité de muscle lisse ainsi qu'une circulation bronchique et des glandes à mucus pauvrement développées (Karol 1994). De plus, les rongeurs ne développent pas de sensibilisation naturelle à des allergènes inhalés. Lorsqu'ils sont sensibilisés expérimentalement, une hyperréactivité bronchique non spécifique est présente mais pas un bronchospasme spontané comme dans une crise d'asthme. L'hyperréactivité est également le plus souvent temporaire, et ce, même quand un certain remodelage est induit avec des challenges antigéniques répétés (Bice *et al.* 2000; Johnson *et al.* 2004a). Le souffle a certains avantages comme modèle animal d'asthme. Premièrement, des études d'anatomie comparée ont montré que les poumons des chevaux ressemblent beaucoup plus aux poumons humains que ceux des rongeurs (Magno 1990; McLaughlin *et al.* 1961). De plus, et ceci est particulièrement important dans les études portant sur le remodelage chronique, leur longévité (30-35 ans) se rapproche de celle des humains et ils peuvent être atteints du souffle pendant plus d'une décennie. Le souffle, comme certaines formes d'asthme, est caractérisé par une réponse exagérée des animaux susceptibles à l'inhalation de particules antigéniques inhalées, ce qui entraîne par une inflammation des voies respiratoires inférieures, une obstruction respiratoire réversible et une hyperréactivité bronchique. Cliniquement, les chevaux atteints présentent de la toux, des sifflements (« wheezes ») audibles à l'auscultation thoracique et de la détresse respiratoire lors d'exposition antigénique (**Figure 6**) (résumé par (Robinson 2001)). L'obstruction respiratoire est principalement causée par le bronchospasme (Camargo *et al.* 2007; Derksen *et al.* 1999; Murphy *et al.* 1980; Robinson *et al.* 1993), mais elle est aussi accentuée par l'accumulation de mucus et de cellules inflammatoires, et possiblement à plus long terme, par un remodelage de la paroi des voies respiratoires, notamment du muscle lisse (**Figure 7**) (résumé par (Leclere *et al.* 2011).


**Figure 6. Difficulté respiratoire chez un cheval atteint du souffle.**

L'obstruction des voies respiratoires se traduit à l'examen clinique par un tirage nasal et une excursion exagérée des côtes lors de l'inspiration (A et B), ainsi que d'une pousse abdominale excessive lors de l'expiration (C). Ces signes cliniques sont réversibles avec un retrait de l'exposition antigénique.

L'infiltration inflammatoire péribronchique est surtout mononucléaire, alors que la population cellulaire dominante dans la lumière des voies respiratoires est neutrophilique, comme dans certains phénotypes d'asthme. Les mastocytes présents dans l'épithélium et l'espace péribronchique augmentent après un challenge antigénique (Dacre *et al.* 2007; van der Haegen *et al.* 2005) et relâchent plus d'histamine (Hare *et al.* 1999). Les éosinophiles quant à eux ne se retrouvent qu'en faible quantité dans le liquide de lavage bronchoalvéolaire, et ils n'augmentent habituellement pas chez les chevaux atteints du souffle (Jean *et al.* 2011; Robinson 2001). Ils participent parfois à l'inflammation péribronchique (van der Haegen *et al.* 2005). Les signes cliniques associés au souffle peuvent être contrôlés par la cessation de l'exposition antigénique ou par l'administration de bronchodilatateurs et de corticostéroïdes (Derksen *et al.* 1992; Jean *et al.* 1999; Lapointe *et al.* 1993).

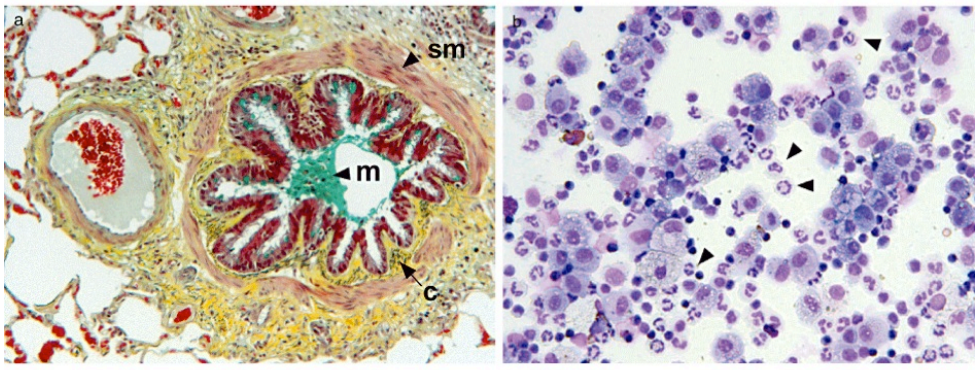



**Figure 7. Changements histologiques et cytologiques dans le souffle.**

A) Bronchiole d'un cheval atteint du souffle en exacerbation clinique. Du mucus (m) et des cellules inflammatoires sont habituellement présents dans la lumière. Les bronchioles sont entourées de muscle lisse (sm), de collagène (c) et de cellules inflammatoires. Coloration Movat Pentachrome, 10X. B) Cellules inflammatoires, incluant de nombreux neutrophiles (flèches noires), retrouvées dans une cytologie de lavage bronchoalvéolaire d'un cheval en exacerbation clinique. Coloration Wright-Giemsa modifiée, 20X. Tiré de Leclere *et al.* Heaves, an asthma-like disease of horses. Respirology. 2011. (Leclere *et al.* 2011).

Les antigènes habituellement mis en cause incluent les fongi, moisissures (McGorum *et al.* 1993), mites (acariens) et possiblement pollen (Lowell 1964) retrouvés en grande quantité dans le foin (Robinson 2001). Les endotoxines, également trouvées en quantité importante dans les écuries, accentuent la réponse inflammatoire mais ne sont pas suffisantes en soi pour reproduire la maladie, et elles induisent une inflammation sans signes cliniques chez les chevaux sains (Pirie *et al.* 2003a; Pirie *et al.* 2003b). Comme dans l'asthme, une susceptibilité génétique a été décrite (Marti *et al.* 1991; Ramseyer *et al.* 2007) et une composante allergique est supportée par la présence d'immunoglobulines E (IgE) dirigées contre certains antigènes dans les voies respiratoires ou le sérum de chevaux atteints

(Kunzle *et al.* 2007; Schmallenbach *et al.* 1998a; Schmallenbach *et al.* 1998b), ou encore par la présence accrue de mastocytes chymase$^{+}$ dans l'épithélium et la *lamina propria* péribronchiolaires (van der Haegen *et al.* 2005). Bien que toutes les études sur le sujet n'aillent pas dans le même sens, certains auteurs ont rapporté que la maladie est associée à un profile type Th2 (Cordeau *et al.* 2004; Horohov *et al.* 2005; Lavoie *et al.* 2001) et un polymorphisme du récepteur à l'interleukine 4 est associé à la maladie dans certaines familles (Jost *et al.* 2007). Les principales similitudes et différences entre le souffle et l'asthme sont résumées dans la **Tableau III** et une revue exhaustive du souffle comme modèle d'asthme est disponible en annexe (Annexe 1) (Leclere *et al.* 2011).

| **Tableau III. Similarités et différences entre le souffle et l'asthme.** | | |
|---|---|---|
| | Asthme | Souffle |
| Pathophysiologie | | |
| Bronchospasme réversible | ✓ | ✓ |
| Hyperreactivité bronchique | ✓ | ✓ |
| Aiguë | ✓ | |
| Retardée | ✓ | ✓ |
| Inflammation éosinophilique | ✓ | |
| Inflammation neutrophilique | ✓* | ✓ |
| Inflammation mastocytaire | ✓ | ✓ |
| Réponse spécifique IgE | ✓* | ? |
| Effet « potentiateur » des endotoxines | ✓ | ✓ |
| Profil inflammatoire | | |
| Th2 et/ou Th1 | ✓ | ✓ |
| Th17 | ✓† | ✓ |
| Site de l'inflammation | | |
| Voies respiratoires périphériques | ✓ | ✓ |
| Voies respiratoires centrales | ✓ | ✓ |
| Remodelage tissulaire | ✓ | ✓ |
| Traitements pharmacologiques | | |
| Corticostéroïdes | ✓ | ✓ |
| Agonistes $\beta_2$-adrénergiques | ✓ | ✓ |
| Anti-leucotriènes | ✓ | |

* : certains phénotypes seulement, † : phénotypes sévères

? : pas de consensus ou données contradictoires

Adapté de Leclere *et al.,* Heaves, an asthma-like disease of horses. Respirology. 2011. (Leclere *et al.* 2011).

**Évidence de remodelage musculaire péribronchique chez les chevaux atteints du souffle**

Le remodelage pulmonaire décrit chez les chevaux atteints du souffle inclut le détachement et la régénération épithéliale, l'hyperplasie des cellules à gobelet et l'augmentation du muscle lisse péribronchique (Herszberg *et al.* 2006; Range *et al.* 2007). Il existe dans la littérature vétérinaire plusieurs mentions de la présence d'hypertrophie ou d'hyperplasie du muscle lisse péribronchique chez les chevaux atteints du souffle. En 1973, Gerber décrit de façon subjective une hyperplasie du muscle lisse (Gerber 1973). En 1983, dans sa thèse doctorale "Structural-functional correlations of the lung in horses with small airway disease", Viel observe une hypertrophie musculaire chez les chevaux avec des signes cliniques sévères. Il utilise un score semi-quantitatif qui tient compte du nombre de couches de myocytes et de la continuité du muscle autour des voies respiratoires (Viel 1983). Plus récemment, Herszberg et collègues (2006) ont utilisé des techniques morphométriques pour quantifier le muscle lisse péribronchique sur des spécimens post-mortem. Ils ont montré une augmentation significative du muscle lisse chez les chevaux atteints du souffle, surtout dans les voies périphériques (Herszberg *et al.* 2006).

# Réversibilité du remodelage du muscle lisse

## Approches thérapeutiques dans l'asthme et le souffle

De façon générale, les approches thérapeutiques dans le souffle et l'asthme visent à contrôler les exacerbations cliniques. Les principales approches sont décrites dans cette section, alors que l'on s'attardera sur leurs effets sur le remodelage dans les sections suivantes. Le souffle n'est pas considéré comme une maladie qui peut être guérie parce que les animaux atteints continuent de développer des signes cliniques lorsqu'ils sont exposés aux antigènes présents dans le foin. De façon similaire, la médication utilisée dans l'asthme

vise à le contrôler et non à le guérir. (Ohta *et al.* 2011). En effet, les personnes asthmatiques, surtout celles qui sont diagnostiquées après l'enfance, ont tendance à présenter des manifestations cliniques qui persistent ou qui progressent avec le temps (Panettieri *et al.* 2008a). La pierre angulaire du traitement du souffle est la réduction de l'exposition antigénique. Les corticostéroïdes et les bronchodilatateurs sont administrés pour diminuer rapidement le bronchospasme et l'inflammation, ou lorsque les modifications environnementales ne permettent pas de diminuer suffisamment l'exposition antigénique (Robinson 2001). Dans l'asthme, le contrôle environnemental est également une composante importante du traitement à long terme, mais il s'avère plus difficile d'éviter l'exposition antigénique. Les corticostéroïdes et les bronchodilatateurs administrés par inhalation restent les médicaments les plus utilisés et les plus efficaces (EPR-III 2007).

**Réduction de l'exposition antigénique**

Dans le souffle, comme dans l'asthme allergique, la cessation de l'exposition aux antigènes inhalés est centrale dans le traitement de la maladie (EPR-III 2007; Jean *et al.* 1999). Il a été montré à maintes reprises que mettre les chevaux au pâturage, sans aucun accès à du foin, permet de contrôler les signes cliniques et entraîne la quasi normalisation de la fonction respiratoire ainsi que la résolution de la neutrophilie pulmonaire (Couetil *et al.* 2005; DeLuca *et al.* 2008; Dixon *et al.* 1995; Jean *et al.* 1999; Leclere *et al.* 2010a). Les signes cliniques s'estompent en quelques jours ou quelques semaines, selon la sévérité et la durée de la maladie (Thomson and McPherson 1984), mais une obstruction respiratoire sous clinique peut persister (Miskovic *et al.* 2007). Cette obstruction sous clinique est surtout observée lorsque le contrôle environnemental se fait en gardant les animaux à l'intérieur et en remplaçant le foin par du fourrage sous forme de cubes ou de pellets (Miskovic *et al.* 2007; Vandenput *et al.* 1998; Votion *et al.* 1999). Chez les patients asthmatiques, un contrôle strict de l'exposition antigénique est souvent difficile à mettre en place, mais lorsque mis en place, il entraine une amélioration de la fonction pulmonaire et une diminution de l'inflammation (Milanese *et al.* 2004; Peroni *et al.* 2002; Popplewell *et al.* 2000), indépendamment de l'administration d'autres thérapies.

## Corticostéroïdes

*Mécanismes d'action des corticostéroïdes*

Les corticostéroïdes sont les principaux anti-inflammatoires utilisés dans le traitement du souffle et de l'asthme. Ils agissent principalement en se liant aux récepteurs (GR) cytoplasmiques des cellules inflammatoires. Dans l'asthme, ceci inclut les éosinophiles, les mastocytes, les neutrophiles et les lymphocytes. Les neutrophiles humains sont parfois considérés comme étant relativement résistants aux corticostéroïdes (Belvisi 2004). Une fois les corticostéroïdes liés à leurs récepteurs, il y a translocation des complexes ainsi formés au niveau du noyau et liaison à l'ADN de la cellule. Les actions génomiques incluent la transactivation, la transrépression et la liaison à des éléments qui inhibent la transcription, soit directement, soit par compétition (Buttgereit *et al.* 2004; Schacke *et al.* 2002). Dans l'asthme et le souffle, la majorité des effets bénéfiques sur la fonction pulmonaire sont attribués à leurs effets anti-inflammatoires, via la transrépression de molécules inflammatoires telles l'IL-1β et le TNFα, cytokines libérées par les monocytes/macrophages et autres cellules inflammatoires et structurelles, et qui augmentent la réponse contractile à l'acétylcholine des cellules musculaires lisses (Hakonarson *et al.* 2001; Hakonarson *et al.* 1997). Chez les chevaux, les corticostéroïdes ont des effets génomiques classiques sur les neutrophiles circulants, dont la suppression de TNFα, d'IL-8 et du récepteur à l'IL-4 (Lecoq *et al.* 2009). Ils ont aussi des effets anti-inflammatoires non génomiques tels que décrits chez plusieurs types cellulaires de d'autres espèces, incluant des cellules bronchiques humaines (Stellato 2004), et contribuent à diminuer la respiration oxydative des neutrophiles (Lecoq *et al.* 2009), ce qui pourrait avoir un rôle à jouer dans le souffle et dans l'asthme.

*Corticostéroïdes systémiques versus par inhalation*

Les corticostéroïdes administrés par voie systémique ont des effets secondaires qui en limitent l'usage à fortes doses ou de façon prolongée tant chez l'humain (Lipworth 1999)

que chez les animaux. Chez les chevaux, les effets secondaires néfastes qui ont été rapportés incluent la suppression de l'axe hypothalamo-hypophysaire (Dowling *et al.* 1993; Picandet *et al.* 2003; Rush *et al.* 1998b), une altération du métabolisme osseux (Lepage *et al.* 1993), musculaire et hépatique (Cohen and Carter 1992; Ryu *et al.* 2004). Une augmentation de la susceptibilité aux infections a été rapportée (Cutler *et al.* 2001; Edington *et al.* 1985; Mair 1996) et attribuée notamment aux effets des corticostéroïdes sur le système immunitaire à médiation cellulaire et humorale (Burguez *et al.* 1983; Flaminio *et al.* 2007; Slack *et al.* 2000; Targowski 1975).

Malgré l'avancement des connaissances sur les mécanismes d'action des corticostéroïdes, il reste difficile de dissocier leurs effets bénéfiques, qui sont surtout liés à la transrépression de gènes associés à l'inflammation, de leurs effets néfastes, qui sont surtout liés à la transactivation de gènes associés au métabolisme physiologique. Il existe en effet qu'un seul isoforme du récepteur cytoplasmique aux glucocorticostéroïdes qui puisse se lier à ceux-ci (GRα), ce qui rend le développement de glucocorticostéroïdes plus spécifiques difficile. L'isoforme β (GRβ) ne lie pas aux glucocorticoïdes, mais a possiblement un effet inhibiteur négatif en se liant à l'ADN (Barnes 2011). Il existe néanmoins des glucocorticostéroïdes dits « dissociés » qui promeuvent préférentiellement les interactions GR-protéines, qui induisent la transrépression de gènes inflammatoires, alors que les effets néfastes font surtout suite à des interactions directes entre le GR et l'ADN (Barnes 2011; Schacke *et al.* 2007). Une autre approche est de chercher à obtenir des concentrations maximales aux sites d'inflammation tout en minimisant l'exposition des autres organes. L'administration de corticostéroïdes par inhalation permet d'atteindre des concentrations élevées au niveau des voies respiratoires en maintenant des concentrations systémiques faibles. L'introduction des inhalateurs MDI (metered dose inhaler) sous pression, à propulsion aux d'hydrofluoroalkanes (HFA), a permis d'augmenter la déposition pulmonaire, incluant au niveau des voies périphériques, de façon importante comparativement aux inhalateurs à poudre sèche (DPI) ou aux MDI propulsés par

chlorofluorocarbones (CFC) (Leach *et al.* 1998; van den Berge *et al.* 2011). Les corticostéroïdes administrés par inhalation constituent maintenant la médication anti-inflammatoire de première ligne dans le traitement de l'asthme (EPR-III 2007). Chez les chevaux, ils sont utilisés dans le traitement du souffle et ils permettent d'améliorer la fonction respiratoire et de contrôler les signes cliniques (Ammann *et al.* 1998; Couetil *et al.* 2005; Giguere *et al.* 2002a; Robinson *et al.* 2009; Rush *et al.* 1998a). La suppression du cortisol endogène a néanmoins été rapportée et suggère une certaine absorption systémique (Laan *et al.* 2004; Rush *et al.* 1999; Rush *et al.* 1998b). L'étude de Dauvillier et collègues effectuée récemment (en parallèle de l'étude II, Annexe 5) suggère toutefois qu'il n'y a pas d'altération significative de l'immunité innée ou acquise (de type humoral ou à médiation cellulaire) avec la fluticasone (Dauvillier *et al.* 2011). Avant son utilisation dans l'étude II, la fluticasone avait été évaluée dans différentes conditions expérimentales. Une étude a montré que la fluticasone diminue l'inflammation et améliore de façon significative la fonction pulmonaire lorsqu'elle est combinée à une diminution de l'exposition antigénique, comparativement à l'effet de la diminution de l'exposition antigénique seule (Giguere *et al.* 2002b). Dans une autre étude, la fluticasone avait aussi un effet bénéfique sur la fonction pulmonaire, mais seulement chez les chevaux sévèrement atteints (Couetil *et al.* 2005). Plus récemment, deux traitements de courte durée (trois jours) ont été comparés, soit la fluticasone par inhalation et la dexaméthasone par voie systémique. Alors que les deux permettaient de contrôler l'apparition de signes cliniques lors d'exposition antigénique, seule la dexaméthasone permettait d'améliorer rapidement la fonction pulmonaire des chevaux déjà en exacerbation clinique (Robinson *et al.* 2009). Malgré les évidences de leur efficacité et des effets secondaires moins importants, le coût des corticostéroïdes par inhalation et la difficulté d'administration chez certains chevaux en limite l'utilisation à grande échelle et les corticostéroïdes par voie orale restent encore largement utilisés.

## Effets des corticostéroïdes sur le muscle lisse

Les effets des corticostéroïdes ne se limitent pas à leur action anti-inflammatoire. Ils ont de nombreux effets sur la majorité des types cellulaires, incluant des effets directs sur les cellules structurelles. Certains effets sur les cellules musculaires lisses sont particulièrement pertinents dans l'étude de l'asthme et du souffle et incluent une interférence dans la transmission de la voie de signalisation des récepteurs à l'histamine H1 (Hardy *et al.* 1996), une diminution de la concentration intracellulaire du calcium (Tanaka *et al.* 1995) et une diminution de l'expression des récepteurs muscariniques (Nabishah *et al.* 1991). Ces actions ont en commun de diminuer la contractilité des myocytes. Il existe aussi des évidences que les corticostéroïdes facilitent la relaxation du muscle lisse, notamment en contrecarrant la désensibilisation des récepteurs $\beta_2$-adrénergiques (Davies and Lefkowitz 1984). Ils peuvent aussi faciliter la relaxation via une augmentation de l'activité de l'adénylcyclase (Michel *et al.* 1994) ou de la pompe sodium-potassium ATPase (Schramm and Grunstein 1996). Les corticostéroïdes ont également un effet indirect sur la relaxation du muscle lisse en réduisant l'activité de l'enzyme MAP (mitogen-activated protein) kinase p38, qui prévient la relaxation induite par l'oscillation des myocytes durant le cycle respiratoire (Lakser *et al.* 2008).

Outre leurs effets sur la contractilité, les corticostéroïdes peuvent également inhiber la prolifération des myocytes (Burgess *et al.* 2008; Roth *et al.* 2004; Schramm *et al.* 1996; Young *et al.* 1995), action qui pourrait avoir un effet direct sur le remodelage, surtout si celui-ci est partiellement dû à de l'hyperplasie. Il est à noter que la plupart des études mentionnées dans cette dernière section portent sur des cellules en culture ou des sections de muscle stimulées *ex vivo*. Lors d'études *in vivo,* il est difficile de différencier un effet direct des corticostéroïdes sur le muscle lisse d'un effet anti-inflammatoire global. Une de ces études a néanmoins permis de mettre en évidence la prévention de la diminution du

nombre de récepteurs $\beta_2$-adrénergiques par les corticostéroïdes chez des rongeurs (Mak *et al.* 1995).

## Réversibilité du remodelage musculaire dans l'asthme

La réversibilité du remodelage du muscle lisse est l'objet de nombreuses spéculations. Des études suggèrent que les corticostéroïdes ralentissent le déclin de la fonction pulmonaire chez les sujets asthmatiques adultes (O'Byrne *et al.* 2006; Pauwels *et al.* 2003), ce qui suggère indirectement un effet bénéfique sur le remodelage. En se basant sur l'hypothèse générale selon laquelle l'inflammation pulmonaire excessive et prolongée mène au remodelage, on peut effectivement supposer qu'un contrôle efficace de l'inflammation devrait permettre un retour à une architecture normale des voies respiratoires (Durrani *et al.* 2011). Cette supposition n'est cependant pas facile à confirmer chez l'humain, entre autres parce que des raisons éthiques et des difficultés liées à la répétition de prélèvements biopsiques limitent les études longitudinales portant sur l'évolution du remodelage asthmatique. Une étude effectuée sur des patients asthmatiques adultes a toutefois montré une diminution de 60% de la quantité de muscle lisse dans des biopsies transbronchiques suite à un traitement de six semaines avec des corticostéroïdes (Bergeron *et al.* 2005). Cette réversibilité est substantielle mais dans cette étude, la quantité de muscle lisse n'a pas pu être comparée à celle de sujets sains dans les mêmes conditions expérimentales. Ces résultats laissent toutefois penser que l'administration de corticostéroïdes a un effet positif sur le remodelage du muscle lisse des voies périphériques (i.e. voies évaluées dans les biopsies transbronchiques). Une autre étude portant sur des biopsies endobronchiques de patients asthmatiques a montré une diminution du pourcentage de muscle lisse 24h après un challenge antigénique dans un groupe traité avec des corticostéroïdes (Kelly *et al.* 2010). Les résultats de cette étude sont toutefois difficiles à interpréter, étant donné que la diminution est également présente dans le groupe contrôle recevant un placébo, mais absente dans le groupe recevant des corticostéroïdes et un bronchodilatateur. D'autres études ont montré un effet positif de l'administration prolongée de corticostéroïdes sur

l'épaisseur de la paroi des bronches, mesurée par tomodensitométrie (Capraz *et al.* 2007; Lee *et al.* 2004). Cette diminution d'épaisseur était de 10 à 12% et restait au dessus des valeurs des sujets sains dans l'étude de Lee. Dans l'étude de Capraz, la diminution était de 16%, avec des valeurs similaires entre les sujets asthmatiques et sains à la fin de l'étude. L'effet bénéfique d'une thérapie à long terme n'a cependant pas toujours été démontré avec ce type d'études (Kurt *et al.* 2009). De plus, à cause des limites de résolution de la tomodensitométrie, ces études ne permettent pas de mesurer spécifiquement le muscle lisse, ni les parois des voies respiratoires périphériques. Les études sur l'effet de la réduction de l'exposition antigénique par un contrôle environnemental strict sont encore moins fréquentes, notamment à cause de la difficulté de le prescrire comme seul traitement, sans corticostéroïdes. Cependant, l'asthme lié au milieu de travail offre une opportunité d'étudier l'effet de la réduction de l'exposition antigénique sur l'amélioration des symptômes, de l'inflammation et sur le remodelage. Une étude sur des travailleurs asthmatiques effectuée après plus de dix ans de retrait de l'exposition professionnelle a montré que les sujets asthmatiques n'avaient pas plus de muscle lisse que les sujets non asthmatiques dans leurs biopsies endobronchiques (Sumi *et al.* 2007). Cette étude supporte l'existence de l'effet bénéfique du contrôle environnemental sur la réversibilité du remodelage, sans toutefois le prouver, notamment parce qu'en absence de biopsies au moment du diagnostic, la présence de remodelage du muscle lisse à ce moment reste spéculative.

## Réversibilité du remodelage musculaire chez les modèles animaux d'asthme

Chez les rongeurs, les syndromes asthmatiques induits par une sensibilisation intrapéritonéale à un antigène suivie de challenges successifs se caractérisent par une inflammation pulmonaire, de l'hyperréactivité bronchique non spécifique et souvent un ou plusieurs aspects du remodelage pulmonaire observé chez les patients asthmatiques. Chez ces modèles animaux, une intervention pharmacologique peut limiter l'augmentation du

muscle lisse, surtout si l'administration de corticostéroïdes est précoce (Johnson *et al.* 2008) ou combinée avec d'autres traitements anti-inflammatoires (Henderson *et al.* 2006). Cette prévention du remodelage est attribuée au contrôle de l'inflammation et à l'inhibition de certains facteurs de croissance comme le TGF-β (McMillan *et al.* 2005). Dans une étude chez le rat, l'administration de corticostéroïdes prévenait l'inflammation, l'hyperréactivité bronchique et la prolifération des myocytes (Leung *et al.* 2005). Toutefois, les effets différaient selon les corticostéroïdes utilisés, certains étant moins efficaces que d'autres pour prévenir l'hyperréactivité bronchique. Récemment, une étude a aussi démontré que l'administration de corticostéroïdes prévenait l'augmentation de muscle lisse mais que, étonnamment, elle ne prévenait pas l'hyperréactivité bronchique (Siddiqui *et al.* 2010). Jusqu'à ce jour, il n'existait aucune donnée sur la réversibilité du remodelage chez les chevaux, ni sur aucun modèle animal ayant développé du remodelage pulmonaire sur plusieurs années.

Cette revue de la littérature récente montre qu'il reste des zones grises dans l'état actuel des connaissances sur le remodelage du muscle lisse dans l'asthme, soit par manque de données, soit par une abondance de données contradictoires. Il existe également un espace à combler entre les études effectuées chez les rongeurs dans des conditions environnementales et génétiques standardisées, et le remodelage chronique dans l'asthme. Les points majeurs abordés dans ces travaux incluent les mécanismes soutenant le maintien du remodelage musculaire chronique, la possible réversibilité du remodelage établi depuis plusieurs années, et les effets des corticostéroïdes sur le remodelage musculaire, sur l'inflammation ainsi que sur la fonction pulmonaire.

# Objectifs et hypothèses

## Objectif général

Démontrer la réversibilité du remodelage du muscle lisse observé chez les chevaux atteints du souffle.

## Objectifs spécifiques de l'étude I

1. Développer des méthodes permettant l'étude du remodelage musculaire de façon séquentielle dans une étude longitudinale sur des chevaux atteints du souffle.
2. Évaluer l'effet de la stimulation antigénique sur le muscle lisse lorsqu'un certain degré de remodelage est déjà présent.
3. Comparer le remodelage musculaire observé dans des biopsies pleine épaisseur (obtenues par thoracoscopie) et le remodelage observé dans les biopsies partielles (obtenues par bronchoscopie).

## Objectifs spécifiques de l'étude II

1. Déterminer si le remodelage musculaire chronique chez des chevaux adultes est réversible sur une période de un an.
2. Comparer deux interventions, à savoir l'administration de corticostéroïdes par inhalation et la réduction de l'exposition antigénique, sur le remodelage, l'inflammation et la fonction pulmonaire.

## Hypothèses de l'étude I

1. Les chevaux atteints du souffle ont plus de muscle lisse péribronchique que les chevaux sains du même âge et vivant dans les mêmes conditions environnementales.
2. Une stimulation antigénique accentue cette différence en augmentant la masse de muscle lisse chez les chevaux atteints du souffle alors qu'elle n'affecte pas le muscle lisse des chevaux sains.
3. L'hyperplasie des myocytes contribue à l'augmentation de la masse de muscle lisse, via une augmentation de la prolifération.
4. L'augmentation du muscle lisse peut être mesurée à la fois au niveau des voies respiratoires périphériques (via des biopsies pleine épaisseur obtenues par thoracoscopie), et au niveau des voies plus centrales (biopsies partielles de la paroi des voies respiratoires obtenues par bronchoscopie).

## Hypothèses de l'étude II

1. Le remodelage chronique du muscle lisse respiratoire est partiellement réversible par l'administration de corticostéroïdes par inhalation ou par un contrôle environnemental permettant une réduction prolongée de l'exposition antigénique.
2. La combinaison de l'administration de corticostéroïdes par inhalation et de la réduction de l'exposition antigénique accentue la diminution du remodelage musculaire.
3. La réversibilité du remodelage se fait en autres via une diminution de la prolifération des myocytes.

# Méthodologie

## Survol du plan expérimental

La première étude permet de comparer la masse de muscle lisse péribronchique des chevaux atteints du souffle (n= 6) aux chevaux sains (n=5) en absence et en présence d'une exposition antigénique. Des biopsies endobronchiques et des biopsies du poumon périphérique ont été obtenues par bronchoscopie et thoracoscopie, respectivement, avant et après une exposition antigénique d'une durée de 1 mois.

La seconde étude permet de comparer les changements observés dans les biopsies pulmonaires périphériques sur une période d'un an chez des chevaux atteints du souffle qui sont initialement en exacerbation, puis traités soit uniquement en réduisant l'exposition antigénique (n=5), soit avec des corticostéroïdes administrés par inhalation (n=6). Le groupe traité avec des corticostéroïdes est initialement maintenu dans les même conditions ayant menées à l'exacerbation clinique pendant 6 mois (haute exposition antigénique), puis dans des conditions de faible exposition antigénique pour la seconde moitié de l'étude. La fonction pulmonaire est mesurée et des lavages bronchoalvéolaires effectués avant chaque prise de biopsie et à certains points intermédiaires détaillés dans la **Figure 8** et la **Figure 9**. Par crainte des effets secondaires possibles associés à l'administration de corticostéroïdes par voie systémique sur une période de un an, l'étude II a été effectuée avec de la fluticasone administrée par inhalation.

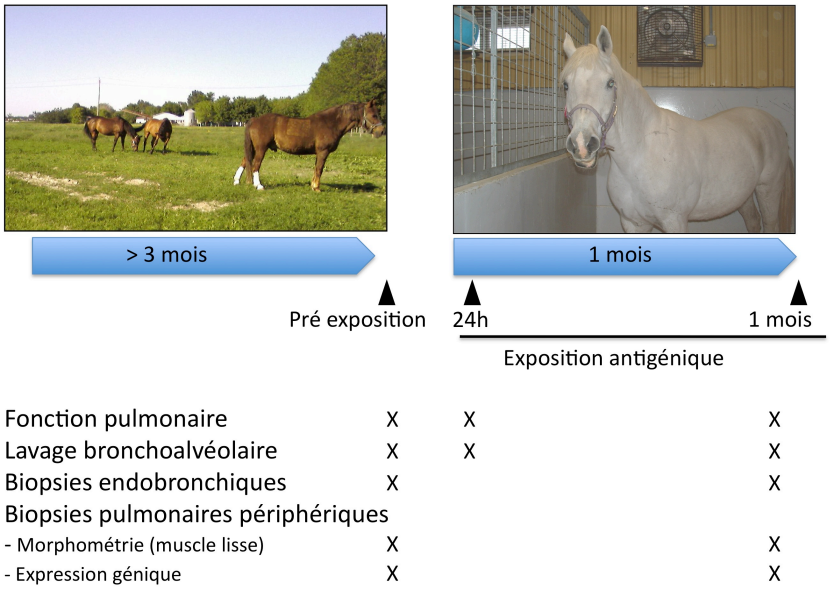



**Figure 8. Plan expérimental de l'étude I.**

Onze chevaux adultes gardés au pâturage (6 chevaux atteints du souffle asymptomatiques et 5 chevaux sains) sont exposés aux antigènes inhalés contenus dans le foin pendant 1 mois. Des prélèvements sont faits pré et post-exposition (24h et 1 mois).

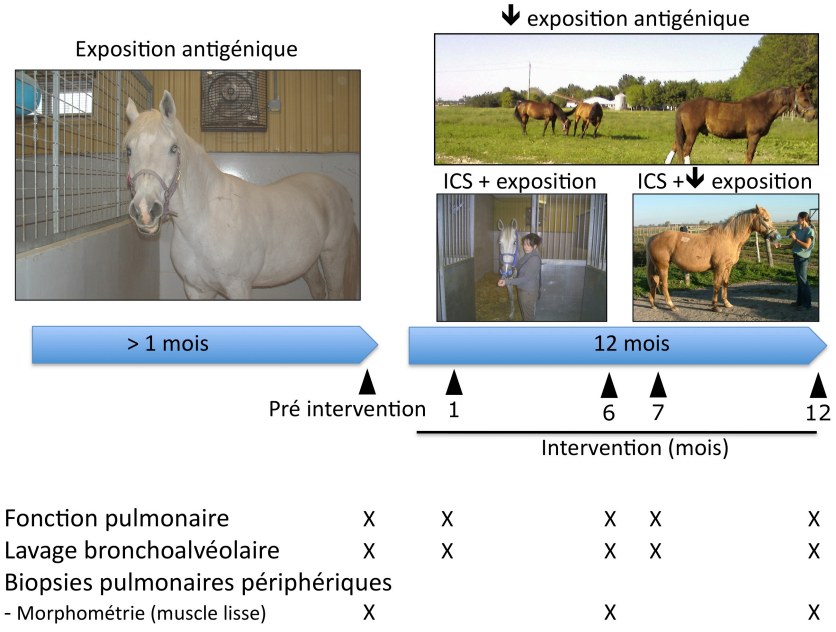



**Figure 9. Plan expérimental de l'étude II.**

Onze chevaux atteints du souffle symptomatiques sont traités soit avec une réduction de l'exposition antigénique seule (n=5), soit avec des corticostéroïdes par inhalation (n=6). Les chevaux traités aux corticostéroïdes sont dans un premier temps (6 mois) gardés dans des conditions de haute exposition antigénique puis, dans la seconde moitié de l'étude, corticostéroïdes et réduction de l'exposition antigénique sont combinés.

## Procédures expérimentales

Les procédures expérimentales sont décrites dans les articles 1 et 2. Certaines procédures centrales au projet sont toutefois peu détaillées dans les articles car elles sont basées sur des publications antérieures. Elles sont donc détaillées dans cette section, en complément de la méthodologie publiée.

## Mesure de la fonction respiratoire

La fonction respiratoire est mesurée ici selon une méthode « conventionnelle » développée chez l'humain et adaptée au cheval. Le principe général est de mesurer le volume d'air déplacé lors d'une respiration (volume courant) et d'approximer simultanément l'effort nécessaire au déplacement de cet air. Le volume courant est calculé à partir du débit d'air mesuré à l'aide d'un pneumotachographe. La pression de part et d'autre du pneumotachographe est mesurée, la résistance du pneumotachographe est connue et constante sur une certaine étendue de valeurs de débit (dans les valeurs physiologiques pour une espèce donnée) et le débit est calculé selon l'équation (Bates 2005):

$$\Delta P_{pt} = R_{pt} \dot{V} \quad \text{ou} \quad \dot{V} = \Delta P_{pt} / R_{pt}$$

Où $\Delta P_{pt}$ est la différence de pression entre l'amont et l'aval du pneumotachographe, $R_{pt}$ la résistance calibrée du pneumotachographe, et $\dot{V}$ le débit d'air. L'intégration du débit dans le temps permet de calculer le volume. Le pneumotachographe doit être chauffé à la température corporelle pour éviter la condensation de la vapeur d'eau contenue dans l'air expiré. La condensation ferait augmenter la résistance de l'appareil et donc faussement diminuer le débit calculé.

La pression transpleurale est estimée par la mesure de la pression œsophagienne dans sa portion thoracique, crânialement au diaphragme et caudalement au cœur. Parce que l'œsophage et l'espace pleural ne sont séparés que par du tissu mou, la pression dans la lumière de l'œsophage, mesurée par un ballonnet, reflète la pression transpleurale. Au repos, la résistance et l'élastance pulmonaire sont calculées à partir de l'équation suivante (Lauzon and Bates 1991) :

$$P_{tp} = R_L \dot{V} + E_L V_T + K$$

Dans laquelle $P_{tp}$ représente la pression transpulmonaire, $R_L$ la résistance, $\dot{V}$ le débit, $E_L$ l'élastance (ou rétraction élastique du poumon), $V_T$ le volume courant et K est une constante correspondant à la pression transpulmonaire à la fin de l'expiration. L'équation linéaire décrite ci-dessus est basée sur une modélisation à un compartiment du système respiratoire dans lequel les voies respiratoires sont représentées par un seul tube et les poumons par un seul réservoir. Dans ce modèle, la résistance est générée par la composante « voie respiratoire » du système (Kessler *et al.* 2001; West 2005), ce qui est bien entendu une approximation de la réalité. En effet, lors de respiration normale, le parenchyme pulmonaire et la paroi thoracique contribuent de façon non négligeable à la résistante totale (Bates *et al.* 1989; Ludwig *et al.* 1987). De façon plus spécifique, chez les chevaux, un pneumotachographe calibré et chauffé à 37°C est connecté sur un masque et associé à un transducteur de pression différentielle. Ce signal est transformé et intégré dans le temps pour calculer le volume courant. Le ballonnet œsophagien est placé de façon à mesurer un maximum de différence de pression transpleurale dans un cycle respiratoire, assez caudalement pour ne pas avoir d'artéfacts cardiaques. Une fois cette position déterminée, elle est prise en note de façon à toujours replacer le ballonnet au même endroit pour un même cheval. Le tube utilisé est également associé à un transducteur de pression (**Figure 10**). La pression transpulmonaire correspond en théorie à la différence entre la pression œsophagienne et la pression atmosphérique. Les signaux des transducteurs de pression sont convertis en signal digital et transmis à un ordinateur équipé d'un logiciel d'analyse (**Figure 10**) qui permet d'obtenir des valeurs de volume courant, fréquence respiratoire, temps expiratoire et inspiratoire, volume minute, résistance et élastance pour chaque respiration, selon l'équation mentionnée ci-haut. De plus, un coefficient de détermination est calculé pour chaque cycle respiratoire en fonction du degré de correspondance entre les données et l'équation de régression multiple décrite précédemment. Le programme permet d'exclure les respirations avec des coefficients inadéquats (habituellement moins de 90%) et surtout les respirations avec des données aberrantes, comme lors de déglutition ou de toux. Idéalement, une dizaine de respirations consécutives sont utilisées pour calculer une

valeur moyenne. Cette méthode a l'avantage d'être assez robuste et répétable chez les chevaux avec des variations circadiennes bien inférieures aux variations entre des sujets sains et des chevaux atteints du souffle symptomatiques (Jean *et al.* 1999). Elle permet aussi de suivre l'effet de traitement et de distinguer des chevaux atteints du souffle symptomatiques et asymptomatiques. Toutefois, cette méthode reste relativement peu sensible pour la quantification de l'obstruction résiduelle chez des chevaux asymptomatiques et les mécaniques conventionnelles des chevaux au pâturage asymptomatiques sont souvent similaires à celles de chevaux sains, alors que des méthodes plus sensibles mettent en évidence un certain degré d'obstruction persistante (Miskovic *et al.* 2007) ou une hyperréactivité bronchique (van Erck *et al.* 2003).

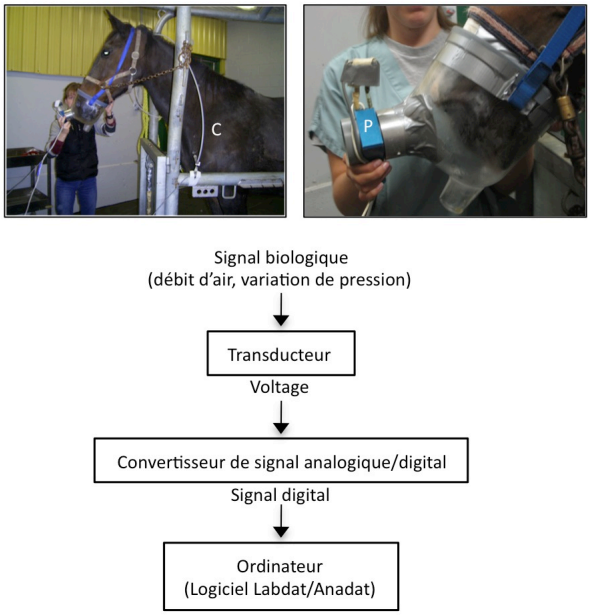



**Figure 10. Mesure de fonction pulmonaire chez un cheval atteint du souffle.**

La pression transpulmonaire est estimée à l'aide d'un cathéter œsophagien (C) placé entre le cœur et le diaphragme et le débit d'air mesuré à l'aide de pneumotachographe (P).

## Lavages bronchoalvéolaires

Le lavage bronchoalvéolaire permet de récupérer les cellules présentes dans la lumière des voies respiratoires distales. Les chevaux reçoivent de la xylazine (agoniste-α2, 0.2 à 0.5 mg/kg, iv) comme tranquillisant et du butorphanol (dérivé opioïde agoniste κ, antagoniste μ, 0.01 à 0.02 mg/kg, iv) comme tranquillisant et antitussif 5 minutes avant la procédure. Un vidéoendoscope (bronchoscope) est avancé dans la bronche principale du poumon gauche ou droit jusqu'à ce que le diamètre de la voie respiratoire empêche sa progression. Deux à trois bolus de 250 ml de saline 0.9 % à 37°C sont instillés et réaspirés immédiatement. Le liquide de lavage est récolté dans des contenants de polypropylène et transporté sur glace pour être cytocentrifugé dans les 2 heures suivant la procédure. Dans les deux études, le côté a été dicté par l'alternance de la thoracoscopie (thoracoscopie et biopsies endobronchiques du même côté et qui alternent dans le temps, lavages bronchoalvéolaires dans le poumon contralatéral). Les comptages différentiels ont été faits sur 400 cellules et les colorations utilisées sont détaillées dans chacun des articles.

## Biopsies endobronchiques

### Procédure

Les biopsies endobronchiques sont effectuées dans le poumon contralatéral, immédiatement après le lavage bronchoalvéolaire. Des pinces sont passées dans le canal du bronchoscope et les biopsies sont prises au niveau de sept à neuf carinae secondaires, aux embranchements accessibles. Les sites les plus distaux sont situés environ 30 cm caudalement à la carina principale. La **Figure 11A** illustre la pince positionnée sur une carina secondaire (Olympus Medical Corps.) et la **Figure 11B** montre une coupe histologique d'une de ses biopsies. Chaque biopsie est placée dans de la formaldéhyde 4% et mise en paraffine 24h plus tard.

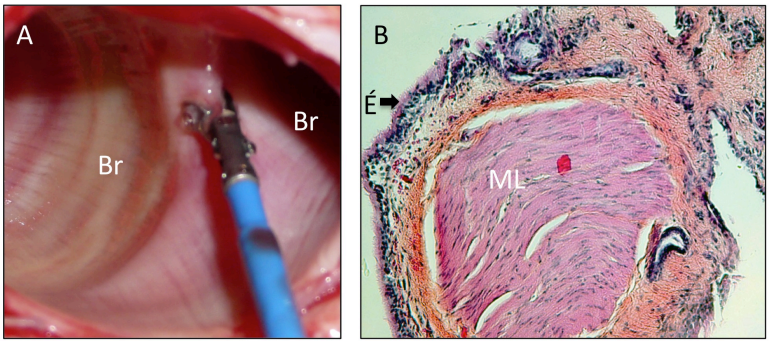



**Figure 11. Biopsies endobronchiques.**

A) Pince à biopsie endobronchique positionnée sur une carina entre deux bronches secondaires (Br). B) Biopsie endobronchique : épithélium (E) et muscle lisse (ML) (Coloration hématoxyline phloxine safran, 10X).

### Morphométrie

De trois à huit (médiane : cinq) biopsies par cheval ont pu être analysées. Sur chaque coupe, le pourcentage de la biopsie occupée par le muscle lisse a été calculé :

(Aire du muscle lisse ($\mu m^2$) / Aire de la biopsie ($\mu m^2$) ) x 100

La densité des myocytes (nombres de myocytes par surface de muscle) a été mesurée en comptant le nombre de noyaux sur une surface de 10 000 à 20 000 $\mu m^2$ de muscle lisse. Le pourcentage de noyaux positifs pour les marqueurs de prolifération et d'apoptose a également été compté sur cette surface.

## Biopsies pulmonaires périphériques

### Procédure

Les biopsies de poumon périphérique ont été effectuées par thoracoscopie, sur des chevaux debout, tranquillisés, respirant de façon spontanée. Cette procédure est relativement peu invasive, comparativement à des biopsies à thorax ouvert, et permet de prendre des

échantillons de façon répétée sur les mêmes chevaux. Les risques et les coûts liés à l'anesthésie générale sont évités, ainsi que l'atélectasie et le barotrauma associé au décubitus et à la ventilation mécanique à pression positive. La technique de biopsie pulmonaire par thoracoscopie a été initialement décrite par Lugo chez les chevaux (Lugo *et al.* 2002). L'utilisation d'agrafes chirurgicales, non résorbables et coûteuses, limite toutefois la prise répétée de biopsies sur un même animal. Une technique dite « au lasso » a été mise au point et utilisée dans le premier article. Brièvement, après une approche de triangulation standard dans les espaces intercostaux 12 à 15, le poumon caudo-dorsal est agrippé par une pince atraumatique et un fil pré-attaché avec un nœud coulant est passé autour de la section de poumon désirée. Le nœud coulant est serré, et la biopsie coupée aux ciseaux. Cette technique s'est avérée rapide et très peu coûteuse, mais nécessite parfois la mise en place d'une seconde boucle, ou l'utilisation d'agrafes. La **Figure 12** illustre la thoracoscopie debout et une vue intrathoracique de la procédure. L'article « Evaluation of a Thoracoscopic Technique Using Ligating Loops to Obtain Large Lung Biopsies in Standing Healthy and Heaves-Affected Horses » (Relave *et al.* 2008) (Annexe 2), décrit la procédure chirurgicale en détails. Dans le second article, un système de cautérisation électrique en une étape a été utilisé. Cette méthode, plus rapide et plus sécuritaire que la méthode au lasso, est décrite dans l'article « Thoracoscopic lung biopsies in heaves-affected horses using a bipolar tissue sealing system » (Relave *et al.* 2010) (Annexe 3). Les deux techniques nécessitent la création d'un pneumothorax unilatéral. Le développement d'un pneumothorax bilatéral est possible et monitoré cliniquement et confirmé par observation du collapse du poumon contralatéral au travers du médiastin dorsal, par la caméra intrathoracique. Dans les cas de pneumothorax bilatéral, une canule d'aspiration est insérée dans le thorax contralatéral afin de rétablir l'expansion de ce poumon durant la chirurgie.

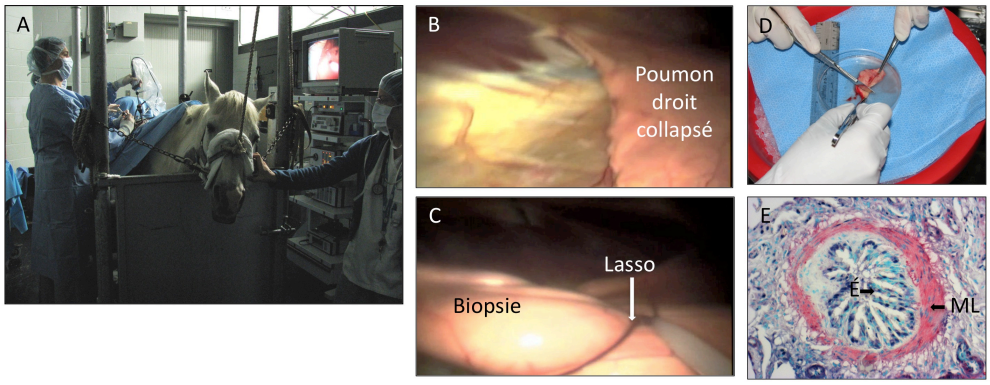



**Figure 12. Biopsies pulmonaires par thoracoscopie**

A) Biopsie pulmonaire par thoracoscopie sur un cheval atteint du souffle. B) Vue intrathoracique du poumon droit après l'induction d'un pneumothorax et la mise en place du thoracoscope. C) Mise en place du lasso. D) Biopsie pulmonaire. E) Coupe histologique d'une biopsie périphérique contenant des voies respiratoires en coup transverse. Muscle lisse péribronchique (ML) et épithélium (É). Muscle coloré par immunohistochimie (α-actin) 10X.

## Morphométrie

Mesurer et comparer la masse de muscle lisse dans des voies respiratoires de différentes tailles et avec différents degrés de bronchoconstriction complique la quantification du muscle péribronchique. Certaines études initiales sur le muscle lisse dans l'asthme ont souffert de ce manque de standardisation (Dunnill *et al.* 1969; James *et al.* 1989; King *et al.* 1999). Le problème a pu être contourné en mesurant le muscle lisse par rapport à la longueur de la membrane basale ou du périmètre interne, tel que décrit par James et al (James *et al.* 1988a; James *et al.* 1988b), afin de corriger pour la taille des voies respiratoires, peu importe le degré de bronchoconstriction. Ceci est possible parce que la membrane basale est considérée comme étant de longueur constante. Il a cependant été démontré plus récemment que la longueur de la membrane basale pouvait être augmentée

lorsque des poumons récoltés post-mortem sont fixés avec des pressions de fixation élevées (McParland *et al.* 2004), ce qui n'est pas le cas dans les études présentées ici.

# Résultats

# Article 1

**Effect of Antigenic Exposure on Airway Smooth Muscle Remodeling in an Equine Model of Chronic Asthma**

Sommaire

Dans cet article, il a été démontré que les chevaux atteins du souffle ont deux fois plus de muscle lisse péribronchique dans leurs voies respiratoires périphériques que les chevaux sains. Ce remodelage est associé à de l'hyperplasie et de la prolifération *in situ,* sans évidence d'une diminution de l'apoptose. Une exposition antigénique additionnelle n'a pas eu d'effet sur la masse de muscle lisse. Dans les voies plus centrales, les myocytes positifs pour les marqueurs de prolifération étaient augmentés, mais seulement après exposition antigénique. Le muscle lisse est donc augmenté mais stable chez les chevaux atteints du souffle de façon chronique. Cette augmentation est maintenue dans un équilibre dynamique par un turnover cellulaire élevé, ce qui suggère qu'une intervention visant à diminuer la prolifération des myocytes pourrait être efficace à faire diminuer le remodelage, même chronique.

**Contribution**

J'ai contribué à l'élaboration des protocoles et à la réalisation de toutes les procédures dont la collecte des données physiologiques (90%), des lavages bronchoalvéolaires (70%), des biopsies endobronchiques (90%), l'immunohistochimie (100%), le marquage enzymatique (10%), la morphométrie (100%), les analyses statistiques (40%), l'analyse des données et la rédaction du manuscrit (90%). Les biopsies pulmonaires par thoracoscopie étant faites par un chirurgien (FD), mon rôle s'étendait de la préparation des chevaux, à la tranquillisation et au suivi postopératoire (80%).

**Article publié**

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EFFECT OF ANTIGEN EXPOSURE ON AIRWAY SMOOTH MUSCLE REMODELING IN AN EQUINE MODEL OF CHRONIC ASTHMA

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Running title: ASM remodeling: effect of antigen exposure

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At a Glance Commentary

Scientific Knowledge on the Subject: Recent studies suggest that airway smooth muscle remodeling is an early event in asthma but it is unknown whether it remains a dynamic process late in the course of the disease and how antigen exposure affects established remodeling.

What This Study Adds to the Field: We showed that a 30-day antigen exposure had little effect on established remodeling in diseased animals, despite the development of inflammation and bronchoconstriction. In peripheral airways, airway smooth muscle remodeling appears to be maintained in a dynamic equilibrium by an elevated turnover with *in situ* proliferation, suggesting that targeting airway smooth muscle proliferation could be effective at decreasing its mass.

## Abstract

Rationale: Recent studies suggest that airway smooth muscle remodeling is an early event in asthma but it is unknown whether it remains a dynamic process late in the course of the disease. Little is known of the effect of an antigenic exposure on chronically established smooth muscle remodeling.

Objectives: To measure the effect of antigen exposure on airway smooth muscle in central and peripheral airways of horses with heaves, a naturally occurring airway disease that shares similarities with chronic asthma.

Methods: Heaves-affected horses (6) and age-matched controls (5) were kept on pasture before being exposed to indoor antigens for 30 days to induce airway inflammation and bronchoconstriction. Peripheral lung and endobronchial biopsies were collected before and after antigen exposure by thoracoscopy and bronchoscopy, respectively. Immunohistochemistry and enzymatic labeling were used for morphometric analysis of airway smooth muscle mass and proliferative and apoptotic myocytes.

Measurements and Main Results: In peripheral airways, heaves-affected horses had twice as much smooth muscle as controls. Remodeling was associated with smooth muscle hyperplasia and *in situ* proliferation without reduced apoptosis. Further antigen exposure had no effect on morphometric data. In central airways, proliferating myocytes were increased compared to controls only after antigen exposure.

Conclusions: Peripheral airway smooth muscle mass is stable in chronically affected animals subjected to antigenic exposure. This increased mass is maintained in a dynamic

equilibrium by an elevated cellular turnover, suggesting that targeting smooth muscle proliferation could be effective at decreasing chronic remodeling.

Words: 240
Key words: peripheral airways, animal model, heaves.

## Introduction

Increased airway smooth muscle (ASM) mass is a prominent feature of asthmatic patients, which may play a central role in allergen-induced bronchospasm and in airway hyperresponsiveness to non-specific agonists [1]. ASM remodeling has been demonstrated in airways of asthmatics whether samples were obtained from lung resection or autopsies specimens [2-5], or from endobronchial biopsies [6-9]. Despite ASM remodeling being considered a target for novel therapy [10, 11], the processes leading to and, to even a greater extent, maintaining ASM thickening in chronic disease are unknown. The question is not trivial, as therapeutic approaches targeting established remodeling would differ if it was the result of ongoing proliferation or a decrease in cellular death. This is of particular interest in small airways where direct therapeutic intervention such as bronchial thermoplasty is not possible [12]. Recent publications have also highlighted the importance of the contribution of small airways in asthma while recognizing the difficulty of their sampling and imaging [13, 14]. Among the advantages of using large animal models is the possibility of repeated sampling of peripheral airways, along with the possibility of controlling their environment and treatment, but without the genetic homogeneity of inbred colonies.

Heaves is a naturally occurring disease of horses, associated with domestication and hay feeding, that shares similarities with asthma, including reversible antigen-induced bronchoconstriction, mucus accumulation and airway inflammation. Ten to twenty percent of adult horses are affected by this condition characterized by episodes of cough, wheeze

and exercise intolerance that can be controlled by environmental management (avoiding the offending indoor antigens, usually organic dust from poorly conserved hay), and/or with corticosteroids and bronchodilators [15]. Similar to certain categories of asthma, intraluminal inflammation is predominantly neutrophilic, despite having been linked to Th2 cytokines in some studies [16, 17]. The features of airway remodeling resemble those of asthma and include epithelial detachment and regeneration, goblet cell hyperplasia, and increased bronchial and bronchiolar smooth muscle [18, 19]. Although caused by antigens in moldy hay, heaves does not resemble extrinsic allergic alveolitis as interstitial fibrosis, alveolitis, lymphocytic bronchoalveolar lavage inflammation and restrictive pulmonary dysfunction are not characteristic of the disease [20, 21]. As in humans, reversibility of established ASM remodeling in adult horses has yet to be demonstrated.

In this study, we hypothesized that 1) heaves-affected horses have greater ASM mass than age-matched controls kept in the same environmental conditions 2) a month-long antigen exposure would further increase ASM mass in heaves-affected horses 3) this greater ASM mass would be at least in part due to hyperplasia and 4) similar changes would be seen in peripheral airways (full thickness biopsies) and endobronchial biopsies (partial sampling of airway wall). To test these hypotheses we exposed heaves-affected horses and controls to poorly cured hay and examined ASM in peripheral and endobronchial biopsies, harvested before and after exposure, by means of morphometric analysis of ASM mass, and markers of proliferation and apoptosis.

## Methods

*Experimental design*

Data were collected while horses had been on pasture for > 3 months (Baseline) and after 1 (pulmonary function and bronchoalveolar lavage only) and 30 days of stabling and exposure to poorly cured hay (Antigen exposure).

*Animals*

Six heaves-affected horses and 5 age-matched controls were studied. Heaves-affected horses had a well-documented 3 to 10-year history of reversible airway obstruction and inflammation upon hay exposure. Horses were deemed otherwise healthy based on physical examination, blood count and biochemistry. Animal manipulations were performed in accordance with the Canadian Council for Animal Care guidelines.

*Pulmonary function*

Pulmonary resistance and elastance are calculated from the flow rates obtained from a heated pneumotachograph attached to a mask and the transpulmonary pressure derived from an esophageal catheter, on unsedated animals [22].

*Bronchoalveolar lavage (BAL)*

Two 250-mL boluses of isotonic saline were instilled in a main bronchi through a 2.5 m bronchoscope (Olympus Medical Systems Corp., Tokyo, Japan) as previously described [16]. Additional information is available in the Online Supplement.

*Endobronchial biopsies*

Biopsies were performed in the contralateral lung after the BAL, using disposable forceps (Olympus Medical Systems Corp., Tokyo, Japan). Biopsies (median 5, range 3-8) were taken from different branching sites starting approximately 30 cm distal to the carina and moving cranially.

*Lung biopsies via thoracoscopy*

Peripheral lung tissue of 8 to 12 $cm^3$ was harvested in the caudo-dorsal region of the lung on standing, sedated animals [23]. Samples were fixed for 24h in 4% formaldehyde and embedded in paraffin.

*Immunostaining and enzymatic labeling*

Immunohistochemical staining was performed for the co-localization of proliferating cell nuclear antigen (PCNA) with smooth muscle-specific α-actin [18]. Apoptosis was detected using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Additional information is available in the Online Supplement.

*Morphometry analysis*

In peripheral lung biopsies, ASM area of airways in cross-section was measured using Image-Pro Plus software (MediaCybernetics, Carlsbad, CA). ASM area (median number of airways per animal: 10; range: 5-17), ASM nuclei (8; 4-17), $PCNA^+$ myocytes (8; 1-17) and $TUNEL^+$ myocytes (7; 4-7) were corrected by the internal perimeter squared to account for variation in airway size [24]. In endobronchial biopsies, ASM area was measured as a ratio of the biopsy area (5; 3-8) and ASM cells ($PCNA^+$ (4; 1-5), $TUNEL^+$ (3; 1-4)) were counted in random fields over 1 to 2 $x10^4$ $\mu m^2$. Measurements were made by one investigator (ML) blinded to group and time point sample collection.

*Statistical analysis*

Group characteristics (age, weight, gender) were analyzed by Mann-Whitney test and physiologic data (function and BAL) by repeated-measure ANOVA with *a priori* contrasts. Morphometric data were analyzed using paired and unpaired two-tailed *t*-tests using the average value of the biopsies per animal. Normality was assessed with the Kolmogorov-Smirnov test and visual inspection of the data. BAL cell counts and mast cell percentage were transformed (log and arcsine square-root, respectively) prior to analysis. The software SAS 9.1 (SAS Institute, Cary, NC) was used and $P < 0.05$ was considered significant.

## Results

*Animals*

Age, weight and proportion of mares in heaves-affected horses were not statistically different from controls (median (range): 16 (15-20) and 14 (11-17) years, 467 (444-515) and 504 (450-555) kg; 4 and 5 mares).

*Lung function*

Under low-antigen exposure conditions, lung function of heaves-affected horses was not different from controls. Antigen exposure induced significant airway obstruction only in diseased animals. Figure 1 shows pulmonary resistance and elastance after 3 months of antigen avoidance (Baseline) and following 1 and 30 days of exposure to indoor antigens.

*Bronchoalveolar lavage*

Antigen exposure induced a significant increase in the percentage of neutrophils and mastocytes in heaves-affected horses after 1 and 30 days, along with a decrease in the percentage of lymphocytes. In controls, there was a transient increase in neutrophils also present at day 1 (Figure 2). In absolute cell counts, there was no significant change over time in the number of total cells, lymphocytes, macrophages or eosinophils recovered from BAL in either group. There was an increase from baseline in neutrophils and mastocytes in both groups after 1 day and in mastocytes after 30 days in heaves-affected horses (Table I).

*Airway smooth muscle in peripheral airways*

Figure 3A illustrates ASM stained by immunohistochemistry in a peripheral airway. Individual airways of heaves-affected horses have increased ASM mass, which was more pronounced in the most peripheral airways (Figure 4). In horses with heaves, mean ASM mass (Figure 5A) and myocytes nuclei per perimeter length (Figure 5B) are approximately 2 fold greater than controls and are unaffected by a 30-day antigen exposure. The greater ASM mass observed is not associated with higher cell density (ASM nuclei per ASM area), but instead with a significantly lower density (Figure 5C). These findings are in support of a contribution of both hyperplasia and hypertrophy to ASM remodeling in peripheral airways, with a greater contribution of the former.

*Proliferation and apoptosis of airway smooth muscle in peripheral airways*

At baseline, both $PCNA^+$ myocytes and $TUNEL^+$ myocytes (illustrated in Figure 3C and 3D) were more numerous in heaves than in controls, which suggests that chronic ASM remodeling is associated with an increase in cellular turnover as both proliferation and apoptosis are increased (Figure 6A and B, baseline). This high turnover was unaffected by the 30-day exposure. In contrast, control animals showed an increase in both proliferative and apoptotic cells with antigen exposure compared to baseline ($PCNA^+$: 2.1-fold increase, $P = 0.01$; $TUNEL^+$: 2.4-fold increase, $P = 0.06$) (Figure 6); this increase remained below the level observed in heaves-affected horses and was not associated with a change in ASM mass (Figure 5A).

*Airway smooth muscle proliferation and apoptosis in endobronchial biopsies*

At baseline, there was no difference between groups in ASM area percentage (Figure 7A, illustrated Figure 3B), proliferative density ($PCNA^+$ cells/ASM area) (7C) and percentage of proliferating airway myocytes (7D), or the apoptosis density ($TUNEL^+$ cells/ASM area) (7E) and percentage of apoptotic myocytes (7F). Only a modest increase in ASM myocyte density nuclei was noted in heaves-affected animals (7B). With antigen exposure, ASM area percentage decreased in heaves-affected horses (i.e. biopsies taken during ongoing bronchospasm) but remained stable in controls. The proliferative density and percentage of proliferating airway myocytes were significantly greater in heaves-affected horses after antigen exposure when compared to controls (Figure 7C and 7D). There was no significant difference between groups and time for the $TUNEL^+$ myocytes (Figure 7E and 7F). Interestingly, approximately 50% of $PCNA^+$ ASM cells were found to be in clusters of 3 or more cells (data not shown).

## Discussion

In the current study, we have examined the effect of antigen exposure on ASM mass, proliferation and apoptosis in peripheral and central airways in mature animals that had pre-existing ASM remodeling and had been through multiple cycles of antigen exposure, bronchospasm and inflammation throughout their life. With this natural challenge, only heaves-affected horses developed airway obstruction and sustained BAL inflammation. In peripheral airways, the important findings were that ASM remodeling appears to have reached a new dynamic equilibrium characterized by a high cellular turnover where ASM mass, myocyte number, proliferation and apoptosis markers are increased compared to controls but unaffected by an antigen challenge. Antigen exposure increased proliferation and apoptosis markers in controls without affecting their ASM mass. In central airways, proliferative myocytes was increased in diseased animals only after challenge, and antigen exposure had no measurable effect in controls. We confirmed the hypothesis by which heaves-affected horses have greater ASM mass than age-matched controls and that this remodeling is maintained in part by hyperplasia. However, a month-long antigen exposure did not further increase ASM mass and we could not correlate the changes seen in peripheral airways to the ones in endobronchial biopsies.

*Inflammation, bronchoconstriction and remodeling*

Exposure to indoor antigens induced marked and persistent airway neutrophilia in heaves-affected horses. The neutrophil percentage in BAL, more so than absolute cell counts, has

proven to be useful in monitoring environment-induced airway inflammation in horses [15] but healthy animals can also develop transient inflammation in similar conditions [25]. It was the case here, albeit to a lesser extent than heaves-affected animals and without the development of concomitant airflow limitation or increased ASM mass. The present study indicates that the transient inflammation resulting from antigen exposure in healthy animals also leads to an up-regulation of ASM turnover (seen in peripheral airways) without being associated to airway obstruction or ASM thickening. Inflammation-induced up-regulation of ASM turnover appears therefore to be part of a normal response in healthy subjects. It does not allow us however, to conclude as to whether ASM remodeling in diseased animals is only the result of a greater and more persistent inflammation or to factors intrinsic to ASM as suggested by *in vitro* studies [26, 27].

*ASM remodeling in peripheral airways and lung function*

Using post-mortem lung samples, Herszberg and colleagues [18] showed that horses with heaves have more ASM in medium and small airways than horses without respiratory disease. In the present study, we confirm and extend these findings by housing age-matched controls in the same environment and by looking at the effect of an antigen exposure on parameters of remodeling. While all the heaves-affected horses had a greater mean peripheral ASM mass than the controls, it was not correlated with pulmonary resistance or elastance when horses were symptomatic (data not shown). This finding is in agreement with the recent report by James and colleagues [5] in which small airways smooth muscle

thickness was not correlated with asthma severity, despite being increased compared to controls. In this study of human asthma using post-mortem specimens, only ASM in medium and large airways was associated with severity. It is unlikely that this finding means that ASM remodeling in small airways is irrelevant, but more likely that clinical signs and severity criteria based on need for therapy (as in James and colleagues study), or conventional pulmonary function assessment correlates better with larger airway bronchospasm. It is also of interest that when horses were asymptomatic (at baseline), no difference in lung function was detectable, despite ASM thickening being present. This is in agreement with mathematical modeling predicting that ASM thickening without concurrent bronchoconstriction has only a mild effect on airway caliber and lung function [28]. More refined techniques to assess function, and especially peripheral airway obstruction, may have shown low-grade persisting airflow obstruction, however.

*ASM remodeling in peripheral airways: contribution of hyperplasia to ASM remodeling*

Horses with heaves have more than twice as much ASM mass and approximately twice as many airway myocytes in their peripheral airways as age-matched controls. This alteration in ASM is associated with an increase in proliferating airway myocytes rather than a decrease in apoptosis. These results are consistent with a previous study on post-mortem equine lung tissue [18]. The relatively greater increase in proliferating myocytes than in ASM mass (4.2-fold vs 2.3-fold at baseline) suggests that *in situ* proliferation accounts for some of the increase in ASM, even if the percentage of proliferating airway myocytes was not

significantly different in peripheral airways (data not shown). The differences in myocytes per airway perimeter squared and per measured surface suggest a contribution of both hyperplasia (increased mass resulting from increased cell number) and cellular hypertrophy. The latter is consistent with the increased cell size described in asthmatics [3, 9, 29], but hypertrophy has not been demonstrated by all [7, 30]. The decrease in myocyte nuclei per unit area could also be attributable to an increase in extracellular matrix deposition within the smooth muscle bundle, as we did not measure the cell size directly. Nevertheless, these phenomena are not mutually exclusive and in asthma, there is evidence of both hypertrophy and hyperplasia [3, 9, 29, 31, 32], along with increased extracellular matrix [33, 34], with possible regional differences within the bronchial tree [3]. Finally, showing that at least part of ASM hyperplasia in chronic ASM remodeling is due to *in situ* proliferation suggests that limiting the proliferative capacity of airway myocytes may be of therapeutic value, even without directly inducing cell death.

*ASM remodeling in peripheral airways: limited effect of antigen exposure in heaves-affected horses.*

ASM remodeling parameters were not affected by a month-long antigen exposure in heaves-affected horses. The lack of further increase in ASM mass or cell number suggests that ASM remodeling may reach a plateau once a certain mass is attained. By showing that ASM remodeling can occur early in the natural progression of asthma [35] and that ASM thickening correlates better with severity than duration, the findings by James and

colleagues [5] indirectly support this concept of plateau or equilibrium in ASM remodeling. This plateau, or dynamic equilibrium, could prevent complete airway obstruction by ASM (a phenomenon not known to occur in heaves or in fatal asthma). Interestingly, antigen exposure was associated with a modest but significant rise in proliferative and apoptotic cells only in control animals, which developed transient inflammation without airflow limitation. Taken together, these results suggest that the normal response to a transient inflammatory event is an increase in myocyte proliferation and an appropriate compensatory increase in apoptosis while more pronounced or persistent inflammation leads to an increase in ASM mass in diseased animals. We postulate that once the ASM mass reaches a new dynamic equilibrium, as in peripheral airways of chronically affected horses, myocytes show an elevated baseline turnover that is no longer affected by antigen exposure. It is also possible that ongoing tissue inflammation persists in asymptomatic heaves-affected horses, and contributes to the ongoing proliferation and apoptosis observed at baseline. It is however difficult to predict if a longer exposure could have led to an alteration in this dynamic equilibrium in heaves-affected horses. In controls, because these animals have spent most of their lives exposed to hay, it is unlikely that longer exposure would have led to an increase in ASM mass.

*ASM remodeling in central airways: bronchospasm affects ASM quantification in equine endobronchial biopsies*

At baseline, ASM area percentage in endobronchial biopsies was not significantly increased in heaves-affected horses. Most studies in human patients have found an increase in ASM area (total or percentage) [6, 8, 9, 35] while a few could not demonstrate a difference between groups [36] or the difference was seen only in severe but not intermittent asthmatics [9]. In the current study, not only ASM area percentage was not elevated but a significant decrease was also observed after antigen exposure. Kelly and colleagues [37] noted a similar decrease 24h after an allergen challenge in asthmatics, which they attributed to a dedifferentiation to myofibroblasts. The decrease of ASM ratio after exposure could indeed represent an increase in other subepithelial components, but we also found that sampling during ongoing bronchospasm made the positioning of the biopsy forceps more difficult and the sampling more likely to be superficial as carinae thicken with constriction. Alternatively, the lengthening of the airways that could occur during hyperinflation [38] could increase the distance of the ASM from the carinae. It is also possible that two dimensional morphometric analysis is not appropriate for ASM quantification in horses using endobronchial biopsies, regardless of their clinical stage because of the smaller size of the forceps relative to the bronchial carina. Since these results suggest that bronchoconstriction could affect morphometric measurements, a bronchodilator could be used at the time of sampling to avoid this possible confounding factor in the future.

*ASM remodeling in central airways: evidence of ASM cell hyperplasia in endobronchial biopsies*

Despite the difficulties in quantifying ASM in endobronchial biopsies, data on proliferating and apoptotic myocytes could still be obtained. The greater proliferation density and percentage of proliferating airway myocytes in heaves-affected horses after antigen exposure without concurrent increase in apoptosis are suggestive of *in situ* proliferation, even if there was no increase in ASM mass demonstrated in these biopsies. The observation that approximately 50% of proliferating myocytes were found in clusters also supports the presence of *in situ* proliferation, possibly by the autocrine feed-forward mechanism described by Johnson and colleagues [39], or due to a localized source of growth factors present. ASM proliferation has only recently been shown to play a role in ASM remodeling in severe longstanding asthmatics [40]. Before this, James and colleagues [41] had found a percentage of $PCNA^+$ myocytes in a similar range to the one detected here (5 to 8%) but with no difference between asthmatics and controls, while others failed to detect proliferation markers (PCNA, Ki67 or cyclin D1) in ASM bundles [9, 42, 43]. It is worth noting however that in our study the difference was significant only after antigen exposure, when subjects were symptomatic and untreated for a prolonged period, which is less likely to occur in asthmatics. The severity of the disease [7] or the use of corticosteroids [42] can also account for some variations but the processes leading to ASM hyperplasia could also differ among species [44].

## Conclusion

In this equine model of chronic asthma, we observed evidence of hyperplasia associated with *in situ* proliferation, as well as possible hypertrophy in remodeled ASM. An antigenic exposure had no effect on morphometric measurements in peripheral airways and little effect on the percentage of proliferating myocytes in endobronchial biopsies in these chronically affected animals. These findings are in agreement with the concept that ASM remodeling is an early event in asthma and remains stable in terms of mass later in life [5, 45]. We conclude that at least in peripheral airways, ASM remodeling reaches a new dynamic equilibrium in which the increased mass is maintained with an elevated turnover. This also suggests that limiting proliferative capacities of airway myocytes can have a therapeutic value, even without directly inducing cell death.

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Online Supplement

*Bronchoalveolar lavage (BAL)*

Differential cell counts on wright-giemsa stained cytospins were obtained from a count of 400 cells (Hema 3, Fisher Scientific, Kalamazoo, MI). Additional slides were stained for mast cells using Toluidine Blue. The solution was prepared by dissolving 400 µl of acetic glacial acid and 0.025 g of Toluidine Blue in 30 ml of absolute ethanol, adding distilled water to make a total volume of 100 ml.

*Immunostaining and enzymatic labeling*

Immunohistochemical staining was performed for the co-localization of proliferating cell nuclear antigen (PCNA) with ASM on 5 µm sections using smooth muscle-specific α-actin mouse monoclonal antibody (clone 1A4, Sigma Immunochemicals, Toronto, ON), PCNA antibody (clone Ab-1, Calbiochem, San Diego, CA), and methyl green counterstaining [18]. Apoptosis was detected using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (ApopTag, Chemicon International, Temecula, CA), following company's protocol. The signal was developed with diaminobenzidine-nickel (Vector Laboratories, Burlington, ON), followed by an eosin and saffron counterstaining.

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## Figures and tables Article 1

**Tableau IV (Table 1 in Article 1). Bronchoalveolar fluid cell counts.**

| | | | Baseline | Antigen Exposure 1 day | Antigen Exposure 30 days |
|---|---|---|---|---|---|
| Total cell count | (x $10^7$) | C | 4.17 (1.45) | 7.13 (2.69) | 7.97 (0.73) |
| | | H | 3.45 (3.59) | 8.82 (6.91) | 5.91 (13.08) |
| Neutrophils | (x $10^7$) | C | 0.04 (0.02) | 1.53 (1.16)$^{\dagger}$ | 0.80 (0.43) |
| | | H | 0.03 (0.06) | 4.28 (4.29)$^{\dagger}$ | 0.90 (1.80) |
| Lymphocytes | (x $10^7$) | C | 2.02 (1.16) | 1.95 (0.97) | 3.69 (1.40) |
| | | H | 2.20 (2.09) | 1.48 (0.97) | 2.00 (4.50) |
| Macrophages | (x $10^7$) | C | 2.11 (0.51) | 3.50 (1.32) | 3.36 (1.28) |
| | | H | 1.21 (1.53) | 2.96 (2.00) | 3.03 (6.78) |
| Eosinophils | (x $10^5$) | C | 0.00 (0.00) | 1.09 (1.49) | 3.42 (5.56) |
| | | H | 0.09 (0.21) | 0.95 (1.58) | 0.02 (0.06) |
| Mastocytes | (x $10^5$) | C | 0.00 (0.00) | 1.08 (1.17)$^{\dagger}$ | 0.00 (0.00) |
| | | H | 0.01 (0.01) | 5.65 (6.61)$^{\dagger}$ | 0.46 (0.73)*$^{\dagger}$ |

Bronchoalveolar fluid cell counts (mean (SD)). C: Controls, H: Heaves. *: Different between groups at one time point. $^{\dagger}$: Different from baseline within the same group. $P < 0.05$.

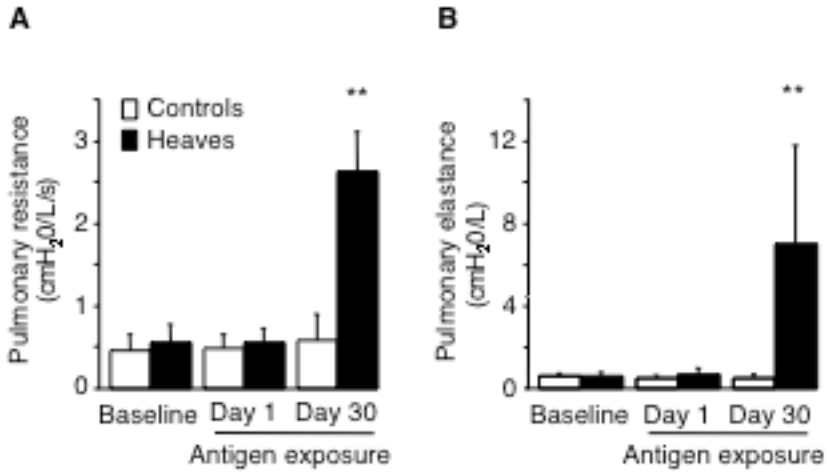


**Figure 13 (Figure 1, article 1). Pulmonary function.**

Pulmonary resistance (A) and elastance (B) following 3 months of antigen avoidance (Baseline) and after 1 and 30 days of antigen exposure. Heaves-affected horses: n=6. Controls: n=5. Mean ± SD. **: Different from Baseline and Day 1 within the same group and different from controls at the same time point. $P < 0.01$.x

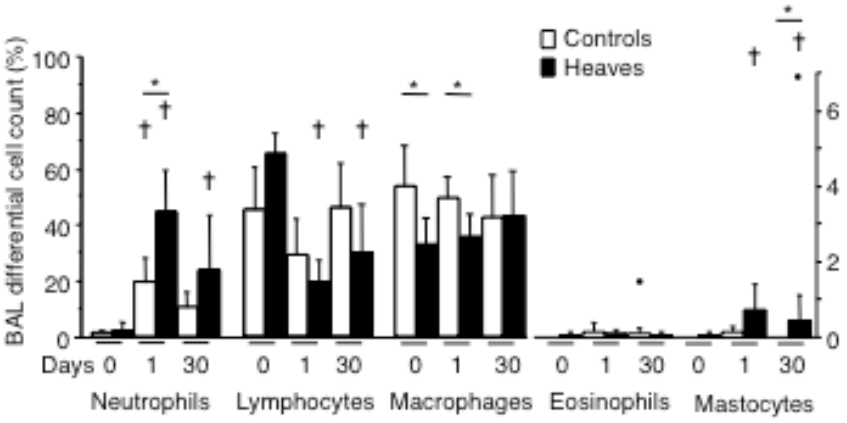


**Figure 14 (Figure 2, Article 1). Bronchoalveolar lavage.**

Bronchoalveolar lavage (BAL) differential cell count following 3 months of antigen avoidance (Baseline = Day 0) and after 1 and 30 days of antigen exposure. Heaves-affected horses: n=6. Controls: n=5. Mean ± SD. *: Different between groups at one time point. †: Different from baseline within the same group. $P < 0.05$. □ : Outlier.

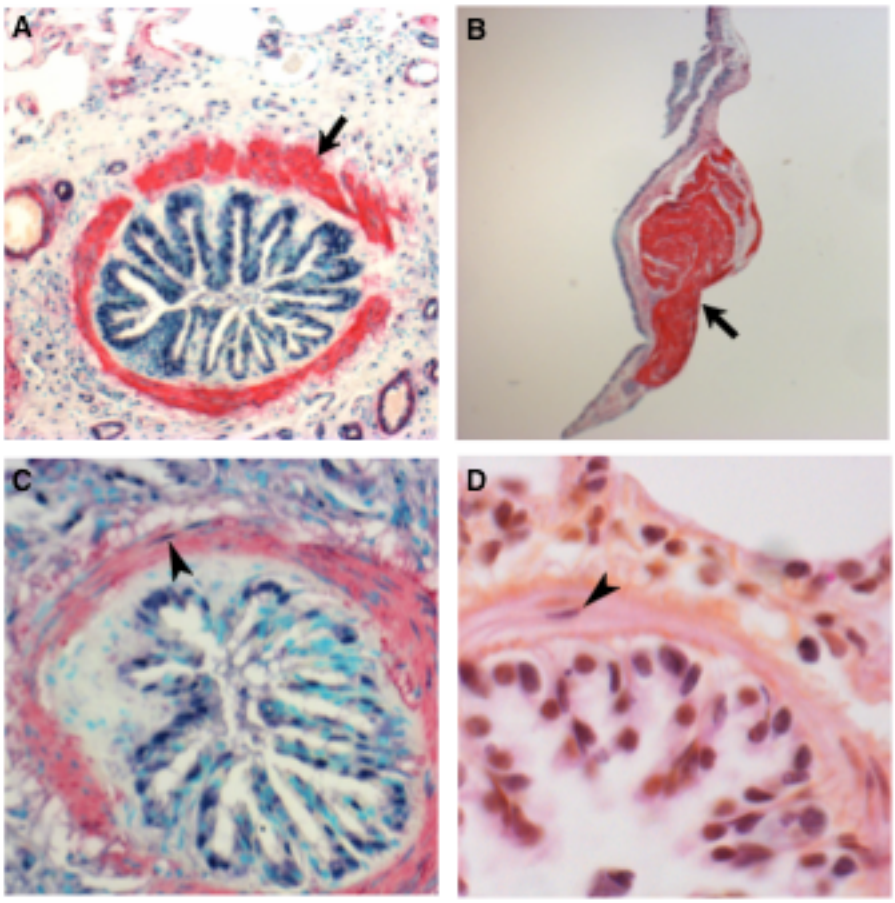


**Figure 15 (Figure 3, Article 1). Airway smooth muscle.**

Airway smooth muscle (arrows) (smooth muscle α-actin stained by immunohistochemistry) in peripheral lung biopsies (A) and endobronchial biopsies (B). Airway myocytes positive for proliferating cell nuclear antigen (PCNA) (C) and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) (D) (arrowheads). Original magnification: 10, 4, 20 and 40X for A, B, C and D, respectively.

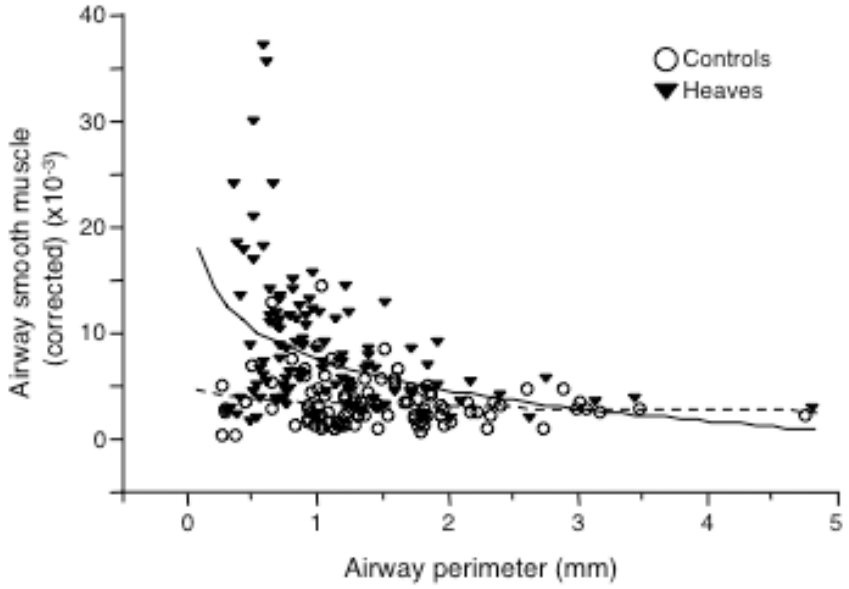


**Figure 16 (Figure 4, article 1). Airway smooth muscle area.**

Airway smooth muscle area corrected for the internal perimeter squared in peripheral airways at both time points. The internal perimeter of the airways in cross section range from 0.2 to 4.8 mm (x axis). Logarithmic regression lines illustrate the trends for diseased animals (full line) and controls (dotted line). Controls: 108 airways. Heaves: 109 airways.

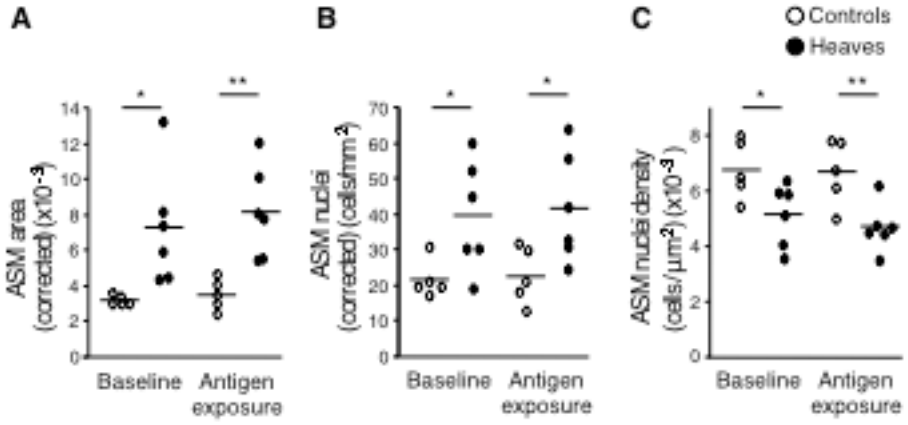


**Figure 17 (Figure 5, article 1). Airway smooth muscle area.**

Airway smooth muscle area (ASM) (A) and ASM myocyte nuclei (B) corrected for the internal perimeter squared, and (C) myocyte density (ASM nuclei / measured ASM area) following 3 months of antigen avoidance (Baseline) and after 30 days of antigen exposure. Heaves-affected horses: n=6. Controls: n=5. — : mean. * $P < 0.05$. ** $P \leq 0.01$.

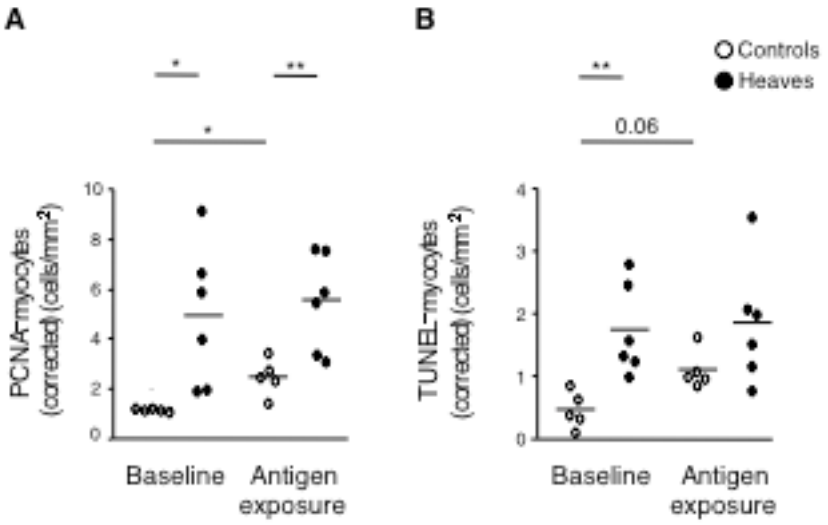


**Figure 18 (Figure 6, article 1). Proliferation and apoptosis.**

Proliferation and apoptosis in peripheral airways. $PCNA^+$ (A) and $TUNEL^+$ (B) myocytes corrected for the internal perimeter squared following 3 months of antigen avoidance (Baseline) and after 30 days of antigen exposure. Heaves-affected horses: n=6. Controls: n=5. ── : mean. * $P < 0.05$, ** $P \leq 0.01$.

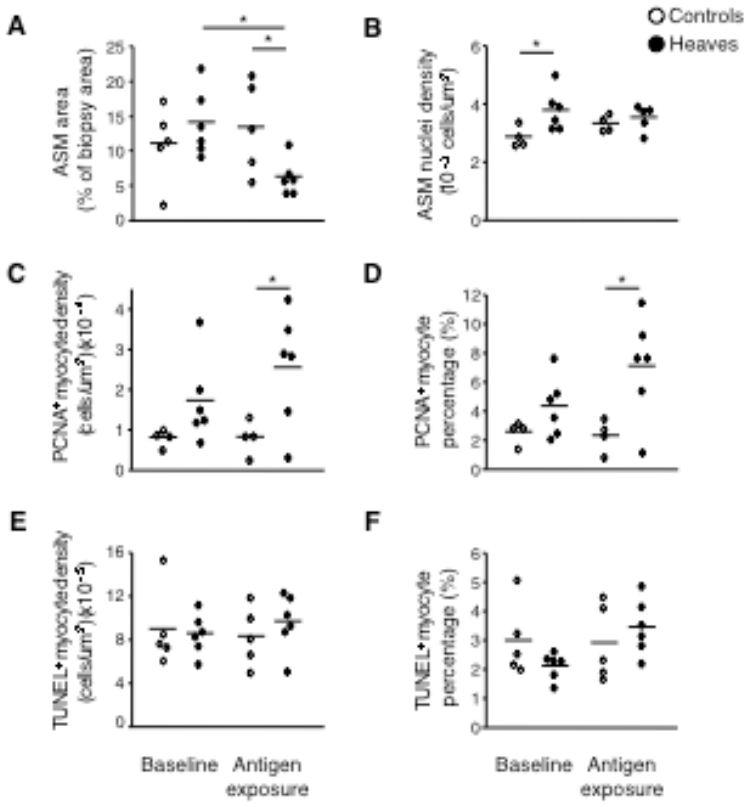


**Figure 19 (Figure 7, article 1). Airway smooth muscle in endobronchial biopsies.**

Percentage of ASM area (ASM area/biopsy area) (A), myocytes density (myocytes nuclei/ASM area) (B) $PCNA^+$ myocytes density ($PCNA^+$ myocytes/ASM area) (C), percentage of $PCNA^+$ airway myocytes (D) $TUNEL^+$ myocytes density ($TUNEL^+$ myocytes/ASM area) (E), percentage of TUNEL+ airway myocytes (F) in endobronchial biopsies. Biopsies were taken following 3 months of antigen avoidance (Baseline) and after 30 days of antigen exposure. Heaves-affected horses: n=6. Controls: n=5 (except for C and D n = 4). ── : mean. * $P < 0.05$.

# Article 2

**Corticosteroids and Antigenic Avoidance Decrease Airway Smooth Muscle Mass in an Equine Asthma Model**

Sommaire

Cette étude décrit pour la première fois l'effet d'une intervention tardive (i.e. sur des animaux matures malades depuis plusieurs années) sur le remodelage du muscle lisse péribronchique dans le tissu pulmonaire périphérique. L'objectif est de mesurer l'effet des corticostéroïdes administrés par inhalation et de la réduction de l'exposition antigénique sur le remodelage du muscle lisse chez les chevaux atteints du souffle. Onze chevaux symptomatiques ont été traités avec des corticostéroïdes (n = 6) (avec et sans exposition antigénique concurrente) ou uniquement par une réduction de l'exposition antigénique (n = 5). Sur une période d'un an, la diminution du remodelage du muscle lisse fut similaire dans les deux groupes. Cette diminution d'environ 30% a toutefois été plus rapide chez les chevaux traités aux corticostéroïdes, ce qui suggère que l'administration de corticostéroïdes pourrait accélérer la réversibilité du remodelage musculaire lisse. Cette étude suggère toutefois aussi qu'une portion du remodelage chronique pourrait être irréversible.

**Contribution**

J'ai contribué à l'élaboration des protocoles et à la réalisation de toutes les procédures dont la collecte des données physiologiques (fonction respiratoire) (90%) et des lavages bronchoalvéolaires (70%), l'administration des traitements (40%), l'immunohistochimie (100%), le marquage enzymatique (10%), la morphométrie (100%), les analyses statistiques (40%), l'analyse des données et la rédaction de l'article (90%). Les biopsies pulmonaires par thoracoscopie étant faites par un chirurgien (FR), mon rôle consistait à préparer et tranquilliser les chevaux, coordonner le travail des équipes et assurer le suivi postpostératoire (80%).

**Article soumis pour publication**

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CORTICOSTEROIDS AND ANTIGEN AVOIDANCE DECREASE AIRWAY SMOOTH MUSCLE MASS IN AN EQUINE ASTHMA MODEL

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Running title: Chronic ASM remodeling reversibility

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## Abstract

Rationale: Recent studies suggest that airway smooth muscle remodeling is an early event in the course of asthma. Little is known of the effect of long-term antigen avoidance and inhaled corticosteroids on chronically established smooth muscle remodeling.

Objective: To measure the effect of inhaled corticosteroids and antigen avoidance on smooth muscle remodeling in peripheral airways of horses with heaves, a naturally occurring asthma-like disease.

Methods: Heaves-affected adult horses with ongoing airway inflammation and bronchoconstriction were treated with fluticasone propionate (with and without concurrent antigen avoidance) (n = 6) or with antigen avoidance alone (n = 5). Lung function and bronchoalveolar lavage were performed at multiple time points and peripheral lung biopsies collected before and after 6 and 12 months of treatment.

Results: Lung function normalized in both groups. Over the study period, smooth muscle mass decreased from $12.1 \pm 2.8 \times 10^{-3}$ and $11.3 \pm 1.2 \times 10^{-3}$ to $8.3 \pm 1.4 \times 10^{-3}$ and $7.9 \pm 1.0 \times 10^{-3}$ in the antigen avoidance and fluticasone groups, respectively ($P = 0.03$). At 6 months, smooth muscle was significantly lower compared to baseline only in the fluticasone treated animals.

Conclusions: Over the study period, airway smooth muscle remodeling decreased by approximately 30% in both groups, albeit the decrease was faster in horses receiving inhaled corticosteroids. Inhaled corticosteroids may accelerate the reversal of smooth muscle remodeling.

Keywords: Airway remodeling, smooth muscle, inhaled corticosteroids, fluticasone, animal model

## Introduction

Airway smooth muscle (ASM) thickening contributes to the loss of lung function and the development of persistent airflow limitation in asthma, by reducing baseline airway caliber and exaggerating airway narrowing during bronchospasm (1-3). The finding that ASM thickness is related to the severity and not the duration of asthma (2), combined with the increased ASM found in asthmatic children (4) indicate that ASM remodeling could be an early event in the course of the disease and be stable over extended periods of time. However, there is also evidence that ASM remodeling is dynamic even in long established severe asthma (5). Therefore, investigating the effect of intervention late in the remodeling process is important for adult asthmatics, as smooth muscle behavior could differ from what is observed in acutely induced ASM remodeling models. Unfortunately, reversibility of remodeling in people is difficult to study, for ethical reasons and limitations imposed by sampling technique. Recently, a positive effect of a short course of inhaled corticosteroids (ICS) on smooth muscle was described in asthmatics (6). There is also indirect evidence of possible ASM remodeling reversibility with antigen avoidance in work-related asthma (7).

Heaves is a naturally occurring disease of adult horses which shares numerous similarities with asthma, including reversible bronchoconstriction and airway inflammation when susceptible horses inhale antigens present in hay (8, 9). Coughing, wheezing and exercise intolerance are present during clinical exacerbation and can be controlled by antigen avoidance (10, 11), or corticosteroids and bronchodilators (8, 12, 13). Despite having been linked to Th2 cytokines in some studies, intraluminal inflammation is predominantly

neutrophilic, as may also be observed in some types of asthma (14-16). Airway remodeling in heaves includes epithelial detachment and regeneration, goblet cell hyperplasia, and increased bronchial and bronchiolar smooth muscle (11, 17, 18). We have also recently observed that chronic ASM remodeling is maintained in a dynamic equilibrium with an elevated myocyte turn-over (11), not unlike the findings in severe asthma (5). This suggests that an intervention affecting ASM proliferation could reverse ASM remodeling, even if chronically established.

The purpose of this study was to evaluate the effect of long-term treatment with inhaled corticosteroids and antigen avoidance in an equine model of asthma. We hypothesized that 1) chronic ASM remodeling is partially reversible with long-term inhaled corticosteroid (ICS) therapy or prolonged antigen avoidance; and that 2) a combination of both approaches would lead to a greater decrease in ASM. To test these hypotheses we treated symptomatic heaves-affected horses with ICS (with continuous antigen exposure for 6 months, then combined with antigen avoidance) or with antigen avoidance alone. Lung function, inflammation and ASM remodeling in peripheral lung biopsies were measured before, during, and at the end of treatment.

## Material and methods

Additional information is available in the online data supplement (E1).

*Experimental design and animals*

Eleven adult heaves-affected horses were housed indoors and exposed to hay until they all showed clinical signs associated with airflow limitation (Baseline). Treatment consisted of strict antigen avoidance as sole therapy for 5 horses. The remaining 6 horses were treated with ICS; they remained exposed to hay for the first 6 months (ICS with antigen exposure), and then were turned out on pasture for the second half of the study (ICS combined with antigen avoidance). Pulmonary function, bronchoalveolar lavage and peripheral lung biopsies were collected when horses had been exposed to hay for > 1 month (Baseline) and after 6 and 12 months of treatment. Additional pulmonary function and bronchoalveolar lavage data were collected at 1 and 7 months.

*Treatments*

Antigen avoidance consisted in keeping horses on pasture and supplementing them with a pelleted diet. In the ICS group, fluticasone propionate (Flovent® HFA, GlaxoSmithKline, Montreal, Canada) was administered at a dosage of 2000 µg q12h for a month, then dosages were adjusted to control clinical signs (2000 to 3000 µg q12 to 24h). Two horses received 2000 µg q24h from month 1, the other ones requiring higher or more frequent administration during the first 6 months.

*Pulmonary function*

Pulmonary resistance and elastance were measured as previously described (19) (E1). After the last function measurement, a short-acting bronchodilator was administered to evaluate the presence of residual bronchoconstriction. Pulmonary function was measured 30 min later.

*Bronchoalveolar lavage fluid collection*

Bronchoalveolar lavage fluid was collected as described previously (14) (E1).

*Lung biopsies via thoracoscopy*

Peripheral lung tissue was harvested via thoracoscopy in the caudo-dorsal region of the lung on standing, sedated animals (20) (E1).

*Morphometry*

ASM area of airways in cross-section was measured using Image-Pro Plus software (MediaCybernetics, Carlsbad, CA). ASM area (median number of airways per animal: 10; range: 5-17), $PCNA^+$ myocytes (5; 3-5) and $TUNEL^+$ myocytes (5; 2-5) were corrected by the internal perimeter squared to account for variation in airway size (21, 22). Measurements were made by one investigator (ML), blinded to study groups and time points.

*Immunostaining and enzymatic labeling*

Online supplement E1.

*Histological scoring*

For histopathological studies, lung tissue was stained with hematoxylin phloxine saffron stain. The histopathological scores were determined by a pathologist with experience in pulmonary pathology who was unaware of the status of the horses. A semi-quantitative scale was used to score tissue quality, inflammation (bronchial/endobronchial, peribronchial, interstitial) and intraluminal mucus accumulation. As a validation, a second evaluator also read the slides blindly and agreement was fair to excellent for all parameters.

*Statistical analysis*

Physiologic and morphometric data were analyzed by a repeated-measure linear model with "group" as a between-subject factor and "time" as within-subject factor. Within and between groups differences were further evaluated using *a priori* contrasts. Pre and post-bronchodilation data were analyzed separately with the same model. Inflammation scores were analyzed with the Cochran-Mantel-Haenszel test for ordinal data. Agreement between biopsy scoring was evaluated by weighted kappa. $P < 0.05$ was considered statistically significant. Online supplement E1.

## Results

*Animals*

Age, weight and proportion of mares were not statistically different between groups (Antigen avoidance (mean ± SD): 17.4 ± 3.6, ICS: 18.7 ± 2.2 years; 483 ± 40 and 485 ± 60 kg; 3/5 and 4/6 mares). In the ICS group, one horse was not available for lung function measurements and BALF at 7 months, and for another one, post-bronchodilation measurements were not obtained for technical reasons.

*Pulmonary function*

Pulmonary function data are shown in Figure 1A. After antigen exposure (Baseline), both groups had similar degree of airflow limitation, with individual resistance values ranging from 2.7 to 3.8 $cmH_20/L/s$ and 2.1 to 4.4 $cmH_20/L/s$ and elastance from 2.1 to 7.0 $cmH_20/L$ and 2.2 to 6.0 in the antigen avoidance and ICS groups, respectively (reference range: resistance < 1 $cmH_20/L/s$, Elastance < 1 $cmH_20/L$ (19)). Pulmonary elastance rapidly decreased and returned to values close to normal in both groups (significantly different from Baseline at all time points). The decrease in resistance was more pronounced in the first month in the ICS group, as it was significantly lower than in the antigen avoidance group at month 1. This improvement was faster but not complete, as an additional decrease was observed when ICS-treated–antigen exposed horses joined the antigen avoidance group at 6 months.

*Pre and post-bronchodilator lung function*

Resistance was significantly lower following bronchodilator administration after 12 months of treatment (Figure 1B), suggesting residual bronchoconstriction in both groups. The only horse that did not show a decrease in resistance was the one with the lowest pre-bronchodilation value.

*Bronchoalveolar lavage fluid*

As a result of progressive resolution of bronchospasm, the percentage of return volume increased during the experiment, affecting absolute cell counts. Analysis of the differential cell count shows that, as expected in horses with heaves, antigen exposure led to a high percentage of neutrophils in Baseline BALF. In the antigen avoidance group, neutrophils had returned to normal (< 10%) by month 6. In the fluticasone group however, pulmonary neutrophilia only decreased once antigen exposure ceased, with a significant drop observed between months 6 and 7 (Figure 2).

*Airway smooth muscle*

Airway smooth muscle mass, normalized for airway size, was similar in both groups at baseline (Antigen Avoidance (mean ± SD): 12.13 ± 6.20 $x10^{-3}$; ICS: 11.26 ± 2.91 $x10^{-3}$) and significantly decreased over the 12-month period with both treatment regimens (8.33 ± 3.12 $x10^{-3}$; 7.86 ± 2.42 $x10^{-3}$) (P = 0.03 in both groups) (Figure 3). In the antigen avoidance group, ASM decreased gradually over the period of the study, reaching a 27.7 ± 9.6 %

change at 12 months. Interestingly, a similar maximal improvement was also seen in the ICS group but it was reached by 6 months, with no further decrease in the second half of the study (27.7 ± 13.3 % at 6 months (P = 0.048), 28.4 ± 9.7 % at 12 months). Analysis of individual data shows that ASM had decreased in 8 horses at 6 months, and in all but one at 12 months.

*Proliferation and apoptosis of airway smooth muscle*

There was no significant change in $PCNA^+$ or $TUNEL^+$ myocytes between the beginning and the end of the study in either group (Figure 3). However, in the ICS group there was a decrease in PCNA that almost reached significance at 6 months (P = 0.07), which corresponds to the steepest decline in ASM mass observed in the study.

*Inflammation score*

Tissue quality was deemed excellent, very good, good and inadequate in 20, 10, 3 and 0 biopsies, respectively. There was no significant change in the bronchial/endobronchial and interstitial inflammation score. For the peribronchial inflammation and intraluminal mucus scores, there was an overall time effect (P = 0.03) only in the antigen avoidance group, with the scores being marginally lower at 12 months (Figure 4).

## Discussion

In the current study, we have examined the effect of inhaled corticosteroids and strict antigen avoidance on the possible reversibility of ASM remodeling. The results indicate that chronic ASM remodeling is partially reversible, even in animals that have experienced multiple episodes of antigen exposure and airway obstruction over a period of many years. A reduction in ASM remodeling was hastened by ICS but the maximal improvement was of the same magnitude in both groups at the end of the 12-month study period. A cumulative effect of ICS and antigen avoidance in further reducing ASM remodeling, as we had hypothesized, was not observed. These results suggest that a component of smooth muscle remodeling may be refractory to intervention. The faster decrease in ASM mass in the ICS group was not associated with a better control of inflammation, as measured by BALF differential cell count or peripheral biopsy scoring.

### *Inhaled corticosteroids accelerate ASM decrease compared to antigen avoidance alone*

Inhaled corticosteroids accelerated the decrease in ASM remodeling, with almost all of the ASM reduction occurring during the first 6 months of the study in this group. It did not however affect the overall improvement over a year period when compared to antigen avoidance alone, and the improvement remains modest (approximately 30%) in both groups, with mean values remaining approximately twice those of historical healthy controls from two previous studies (11, 17). These results contrast with the complete reversibility of induced airway remodeling observed in rodent models when corticosteroids

are administered early (23) or combined with other anti-inflammatory medication (24). The modest decrease in ASM we observed may nonetheless be clinically relevant, as mathematical modeling indicates that variation in ASM mass leads to much greater changes in airway obstruction during bronchoconstriction (many folds) and in airway hyperresponsiveness (25).

Studies addressing reversibility of ASM remodeling with corticosteroids in asthmatics are scarce. In one study, a substantial (60%) decrease in the percentage of transbronchial biopsies occupied by smooth muscle was observed after only a 6-week treatment with ICS (6). While correction for airway size and comparison with controls were not possible, this study nonetheless suggests that ASM mass can be affected by ICS. Similarly, we observed a significant reduction of ASM in the first 6 months of ICS treatment, and because intermediary time points were not available, we cannot exclude the possibility that most of the decrease took place soon after the initiation of therapy. This accelerated decrease is intriguing and unexpected as it occurred despite continued antigen exposure and evidence of persistent airway inflammation. Corticosteroids are potent anti-inflammatory drugs, and airway remodeling is believed to be a consequence of chronic inflammation (26), therefore making it only logical to hypothesize that remodeling reversal would be attained by inflammation control. We did not observe, however, a positive effect of ICS on BALF differential cell count, histological changes in peripheral airways or intraluminal mucus accumulation (Figure 2 and 4C-D). It is possible that other markers of inflammation not measured in our study were suppressed by ICS. For example, there could be differences in

the level of activation of inflammatory cells or direct anti-inflammatory effects on ASM (27). Alternatively, corticosteroids could affect ASM remodeling independently from their anti-inflammatory effect, by decreasing myocyte proliferation (28, 29). In this study, the steepest decrease in ASM, observed in the first 6 month of treatment with ICS, was associated with an almost significant decrease of myocyte proliferation marker at 6 months ($P = 0.07$) (Figure 3B). An increase in apoptosis was not observed in either group and at any time point, making it an unlikely mechanism of remodeling decrease. Alternative explanations for the decrease in ASM (in both groups) include a decrease in myocyte size (via a decrease in growth factors or stretch levels (30)), a decrease in the extracellular matrix (as it contributes to overall ASM mass) (31, 32), or an inhibition of myofibroblast migration. Finally, the lack of further improvement in the second half of the study could be explained by a partially irreversible increase in mass, therefore making a synergistic effect of ICS and antigen avoidance unlikely to be observed with this experimental design.

*Effects of antigen avoidance on inflammation, function and ASM remodeling*

Current recommendations for asthma therapy include environmental control and allergen avoidance strategies (33). Complete avoidance is difficult to achieve but when implemented, improvement in lung function (34, 35) and inflammation (36) is observed. Little is currently known on the effects of antigen avoidance as sole therapy in human asthmatic subjects (37-39) due to ethical considerations. However, in occupational asthma where it is more easily achieved, work cessation is beneficial to lung function (40, 41),

inflammation (42) and possibly remodeling, as Sumi and colleagues found normal ASM content in bronchial biopsies obtained decades after asthmatics had been removed from their work environment (7). This study however did not show that ASM remodeling was present in these workers initially, as biopsies were not taken at the time of diagnosis. In the current study, antigen avoidance alone was associated with a more rapid control of BALF inflammation than ICS, as well as with modest effect in biopsy inflammation scores at 12 month. Also underlying the importance of antigen avoidance, airway inflammation normalized only when it was added to ICS in the treated group, and it further improved airway function in these horses (significant decrease in resistance between 6 and 7 months, Figure 1). In summary, and despite showing a slower effect on ASM remodeling, this study highlights the importance of antigen avoidance in the control of antigen induced airway inflammation and obstruction.

*Different effects of ICS and antigen avoidance on lung function*

While not the primary goal of this study, a few aspects of the effect of ICS and antigen avoidance on pulmonary function are of interest. First, ICS-treated animals had a faster improvement of lung resistance than horses in the antigen avoidance group, despite being continually exposed to the environmental antigens that triggered airflow limitation in the first place. Because we did not observe evidence of a better control of inflammation in the ICS group, this difference could be a direct effect of glucocorticosteroids on ASM contractility (43, 44), possibly via a downregulation of muscarinic receptors (45, 46).

However, as mentioned above, not all aspects of inflammation were investigated and the degree of activation of inflammatory cells could have influenced bronchoconstriction. While persistent low grade airway obstruction, despite long-term antigen avoidance, has been described (10), the residual reversible bronchoconstriction observed at 12 months was not expected. It raises the question of possible low-grade antigenic stimulation and residual inflammation despite strict environmental control and ICS treatment, as this persistent bronchoconstriction was similar in both groups.

*Dissociation between improvement in airway function and BALF inflammation*

The most striking dissociation between functional improvement and cytological evidence of inflammation was observed in the ICS group. Airway inflammation in BALF persisted as long as antigen exposure continued and resolved when it ceased, indicating that the airway neutrophilia was not driven by the ICS, but was uncontrolled by their use. This was not completely unexpected, as the effect of ICS on inflammation in heaves is inconsistent, even when lung function improves (47-50). In a mouse model of induced asthmatic inflammation, a persistent BALF eosinophilia in dexamethasone-treated/antigen exposed mice was also observed (51). This suggests that corticosteroids may not appropriately control airway inflammation when high antigen exposure is maintained. Lack of effect on BALF inflammation could also be in part due to the function-directed dosing, as higher doses may be required to control inflammation and treating based on inflammation markers would result in higher dosages in people (52). A more subtle but maybe more unexpected

dissociation between inflammation and function was also seen in the antigen avoidance group. While BALF neutrophilia had not changed at 1 month, resistance was significantly lower than at Baseline, and elastance had already normalized. It is possible that the heterogeneity of ventilation improved despite persistence of inflammation due to a decrease in mucus accumulation or in airflow limitation in peripheral airways. Overall, these observations support the notion that antigen avoidance and ICS are complementary, and that treatment efficacy should be monitored not only in terms of function but also inflammation.

## Conclusion

In this study, ICS accelerated the decrease in ASM remodeling when compared to antigen avoidance alone. However, this did not influence the total decrease in ASM observed over a year period, and that improvement remained modest. These results suggest that part of ASM remodeling could be refractory to intervention. Nevertheless, we conclude that 1) there is an additional benefit of antigen avoidance (in terms of lung function and inflammation) even when clinical signs are well controlled by ICS and 2) despite a better control of inflammation with antigen avoidance there is an apparent benefit of ICS treatment because of its effect on ASM remodeling, in addition to rapid lung function improvement.

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Online data supplement

Methods

*Experimental design and animals*

All horses had a well-documented history of heaves for more than 4 years, developing reversible airway obstruction and inflammation upon hay exposure, while being clinically asymptomatic with antigen avoidance. Horses were deemed otherwise healthy based on physical examination, blood count and biochemistry. All procedures were performed in accordance with the Canadian Council for Animal Care guidelines.

*Pulmonary function*

Pulmonary resistance and elastance are calculated from the flow rates obtained from a heated pneumotachograph attached to a mask and the transpulmonary pressure derived from an esophageal catheter, in unsedated animals (19). After the last function measurement at 12 month and to evaluate the presence of residual bronchoconstriction, a short-acting bronchodilator was administered (Salbutamol HFA 1000 μg, Ratiopharm Inc., Mississauga, Canada). Pulmonary function was measured 30 min later.

*Bronchoalveolar lavage fluid collection*

Two 250-mL boluses of isotonic saline were instilled in one main bronchus through a 1.6 m bronchoscope (Olympus Medical Systems Corp., Tokyo, Japan) as previously described (14). Total cell count was determined with a hemocytometer. Differential cell counts on Wright-Giemsa stained cytospins were obtained from a count of 400 cells (Hema 3, Fisher Scientific, Kalamazoo, MI). Additional slides were stained for mast cells using Toluidine Blue. The solution was prepared by dissolving 400 µl of acetic glacial acid and 0.025 g of Toluidine Blue in 30 ml of absolute ethanol, adding distilled water to make a total volume of 100 ml. Mast cell percentage was counted on these slides from a count 400 cells.

*Lung biopsies via thoracoscopy*

Peripheral lung tissue of 8 to 12 $cm^3$ was harvested in the caudo-dorsal region of the lung on standing, sedated animals (20). Samples were fixed for 24h in 4% formaldehyde and embedded in paraffin. At the end of the study, 3 horses in the ICS group and 2 in the antigen avoidance group were subjected to euthanasia for unrelated reasons (chronic lameness and limited housing), lung samples of the same size were collected in the same area immediately after euthanasia and analyzed similarly.

*Immunostaining and enzymatic labeling*

Immunohistochemical staining was performed for the co-localization of proliferating cell nuclear antigen (PCNA) with ASM on 5 µm sections using smooth muscle-specific α-actin

mouse monoclonal antibody (clone 1A4, Sigma Immunochemicals, Toronto, ON), PCNA antibody (clone Ab-1, Calbiochem, San Diego, CA), and methyl green counterstaining (11, 17). Apoptosis was detected using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (ApopTag, Chemicon International, Temecula, CA), following the manufacturer's instructions (11). The signal was developed with diaminobenzidine-nickel (Vector Laboratories, Burlington, ON), followed by eosin counterstaining.

*Statistical analysis*

Group characteristics were analyzed by Student's t-test (age and weight) and Fisher's exact test (proportion of mares). Bronchoalveolar lavage cell count, percentage of volume return and differential count were transformed (log transformed for cell count and arcsine square-root for volume and differential) prior to analysis. The software SAS 9.2 (SAS Institute, Cary, NC) was used.

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## Figures Article 2

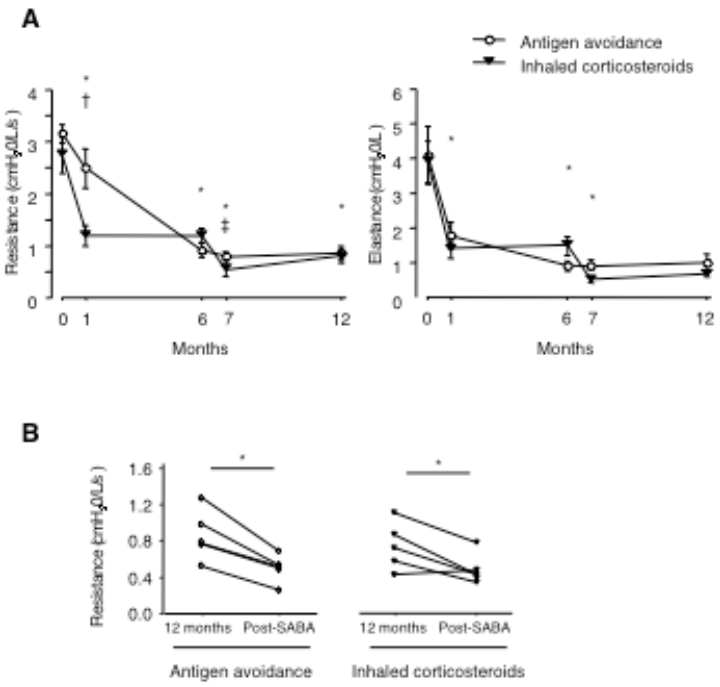


**Figure 20 (Figure 1, Article 2). Pulmonary function.**

1A) Pulmonary resistance and elastance of heaves-affected horses after antigen exposure (Baseline) and after 1, 6, 7 and 12 months of treatment with antigen avoidance alone (open circles) (n=5) or inhaled corticosteroids (black triangles) (n=6). Antigen exposure persisted for the first 6 months of treatment in the ICs group; antigen avoidance was combined to ICS from months 6 to 12. Mean ± SEM. *: Different from Baseline (both groups). †: Different between groups at the same time point. ‡: Different from 6 months (ICS only). $P < 0.05$. 1B) Pulmonary resistance of heaves-affected horses after 12 months of treatment with antigen avoidance alone (open circles) (n=5) or inhaled corticosteroids (black triangles) (n=5), and 30 minutes post administration of a short acting bronchodilator administration (Post-SABA). *: Different between pre and post SABA administration. $P < 0.01$.

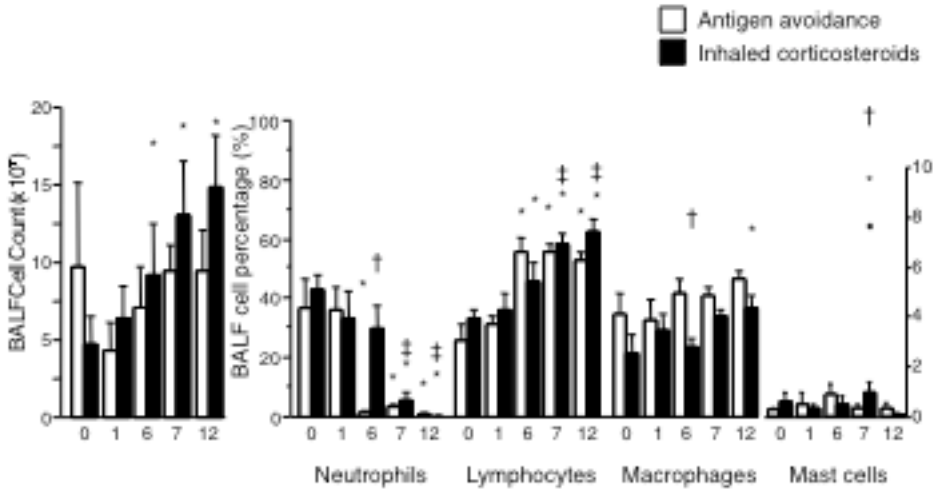


**Figure 21 (Figure 2, article 2). Bronchoalveolar lavage.**

Bronchoalveolar lavage (BALF) total cell count and differential cell count of heaves-affected horses following antigen exposure (Baseline = Month 0) and after 1, 6, 7 and 12 months of treatment with antigen avoidance alone (white bars) (n=5) or inhaled corticosteroids (black bars) (n=6). Eosinophils were absent from all but 6 BALF and are not displayed. Antigen exposure persisted for the first 6 months of treatment in the ICS group. Mean ± SEM. *: Different from Baseline in the same group. †: Different from Antigen Avoidance at the same time point. ‡: Different from 6 months in the same group. □: outlier. $P < 0.05$.

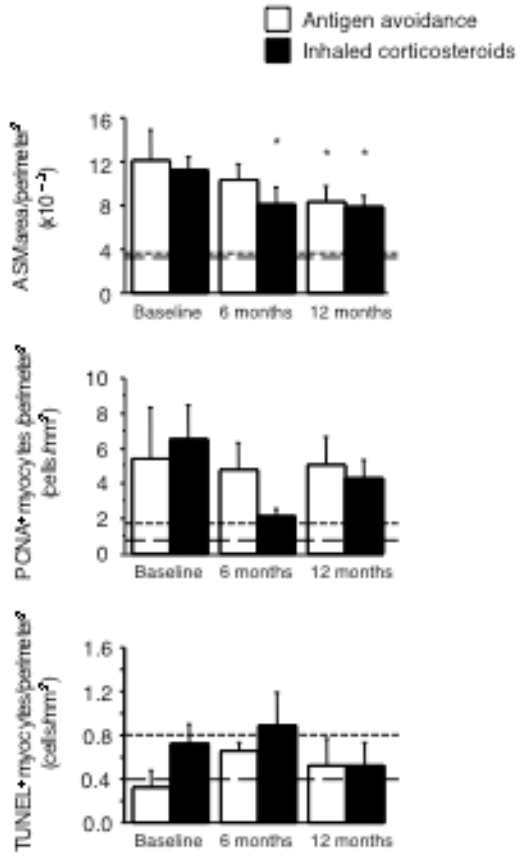


**Figure 22 (Figure 3, article 2). Airway smooth muscle remodeling.**

Airway smooth muscle area (ASM) (A), $PCNA^+$ (B) and $TUNEL^+$ (C) myocytes corrected for the internal perimeter squared of heaves-affected horses following antigen exposure (Baseline) and after 6 and 12 months of treatment with antigen avoidance alone (white bars) (n=5) or inhaled corticosteroids (black bars) (n=6). Antigen exposure persisted for the first 6 months of treatment in the ICS group. Mean ± SEM. Dotted lines represents historical mean values in control horses (− − −: from Herszberg 2006 (17); -----: mean from Leclere 2011 (11)) *: Different from Baseline. $P < 0.05$.

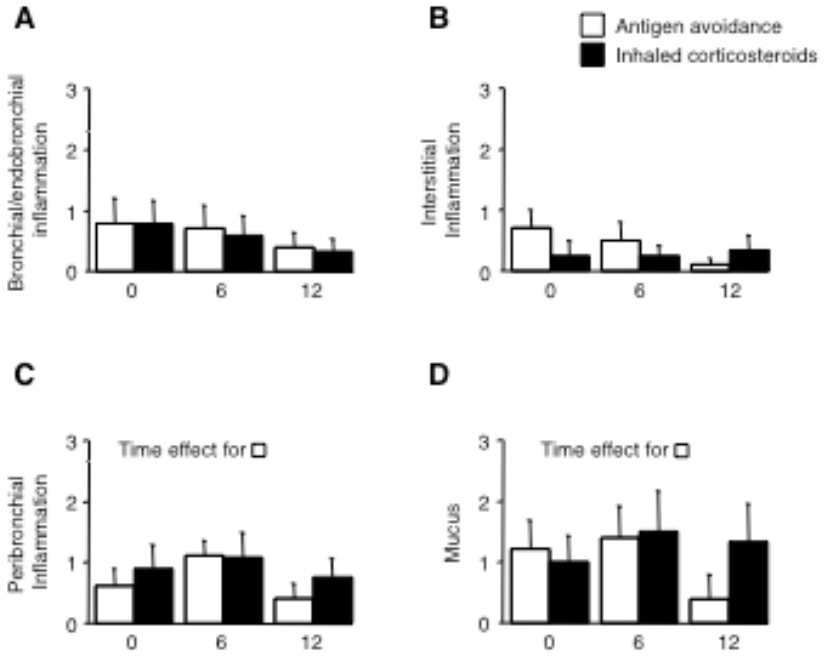


**Figure 23 (Figure 4, article 2). Biopsies histology.**

Tissue inflammation and mucus accumulation in peripheral lung biopsies of heaves-affected horses following antigen exposure (Baseline) and after 6 and 12 months of treatment with antigen avoidance alone (white bars) (n = 5) or inhaled corticosteroids (black bars) (n = 6). Inflammation (bronchial/endobronchial, peribronchial, interstitial) was scored from normal to marked on a 0 to 3 scale. Mucus was scored from 0 to 4. In the antigen avoidance group, there was an overall time effect (P = 0.03) for the peribronchial inflammation (C) and intraluminal mucus scores (D), with the scores being marginally lower at 12 months.

# Discussion générale

L'objectif général des travaux réalisés dans le cadre de cette thèse, à savoir démontrer la possible réversibilité du remodelage du muscle lisse observé chez les chevaux atteints du souffle, a été atteint, tout comme les objectifs spécifiques des études I et II. Ces travaux ont montré que le remodelage du muscle lisse pouvait être mis en évidence dans des biopsies obtenues par thoracoscopie, c'est-à-dire d'une façon relativement peu invasive, qui n'altère pas la qualité de vie des animaux et qui peut être répétée à plusieurs reprises. Cette procédure a permis d'observer l'effet de la stimulation antigénique sur le muscle lisse dans l'étude I et l'effet d'une intervention thérapeutique sur le remodelage dans l'étude II.

*Le muscle lisse péribronchique est augmenté chez les chevaux atteints du souffle, il est approximativement deux fois celui des animaux sains et sa masse n'est pas affectée par une exposition antigénique additionnelle de courte durée.*

Ces conclusions de l'étude I sont importantes sous plusieurs aspects. Tout d'abord, cette étude a permis de confirmer et de renforcer les conclusions d'une étude précédente effectuée sur des prélèvements obtenus post-mortem (Herszberg *et al.* 2006). En effet, dans les deux études, la masse de muscle lisse mesurée chez les chevaux sains est presque identique et l'augmentation observée chez les chevaux atteints du souffle est du même ordre (2.5 à 3 fois plus que chez les chevaux sains). L'étude I a aussi permis de montrer que l'augmentation du muscle lisse chez les chevaux atteints du souffle n'est pas due aux conditions environnementales dans lesquelles les animaux sont gardés et que cette augmentation est présente que les chevaux soient symptomatiques ou non. Lorsque le remodelage du muscle péribronchique est déjà établi, le fait qu'une exposition antigénique additionnelle ne fasse pas augmenter davantage la masse de muscle suggère que le remodelage atteint un plateau ou un niveau de « remodelage maximal ». Cette hypothèse est supportée par certaines observations. Ainsi, le fait que le remodelage musculaire, même sévère, ne mène pas à une oblitération des voies respiratoires (ni dans le souffle, ni dans l'asthme) suggère que l'augmentation de masse du muscle lisse reste sous un certain contrôle physiologique. Il a également été montré récemment dans une large étude

multicentrique que la masse de muscle lisse observée chez des sujets asthmatiques dépend de la sévérité de l'asthme et non de la durée de la maladie (James *et al.* 2009). Cette observation supporte indirectement la présence d'un plateau, puisqu'en l'absence de ce phénomène, on s'attendrait à voir, pour des sévérités similaires d'asthme, une corrélation entre la masse de muscle et la durée. Comme le remodelage est déjà présent chez les très jeunes patients asthmatiques (Tillie-Leblond *et al.* 2008), il semble qu'il pourrait se développer tôt dans la progression de la maladie puis être maintenu dans un équilibre dynamique. L'étude I montre en effet que le remodelage musculaire chronique semble maintenu dans un équilibre dynamique par un turnover cellulaire élevé, dans lequel à la fois la prolifération et l'apoptose des myocytes sont élevées. Des données récentes supportent également cette notion d' « équilibre dynamique » du remodelage musculaire dans l'asthme chez l'humain (Hassan *et al.* 2010). L'implication pour la médecine équine et la recherche sur l'asthme est que si la masse musculaire augmentée est maintenue par un turnover élevé, une intervention thérapeutique diminuant le taux de prolifération cellulaire pourrait avoir un effet positif sur le remodelage, même si celui-ci est chronique.

Un autre aspect important de l'étude I est que, chez les animaux sains, une exposition antigénique induisant une inflammation légère et transitoire (et sans bronchospasme), entraîne une augmentation du taux de prolifération des myocytes. Pourtant, la masse de muscle lisse ne s'en trouve pas affectée, possiblement parce qu'elle s'accompagne d'une augmentation de l'apoptose (augmentation presque statistiquement significative de 2.4 fois, **Figure 18B**). Ces résultats sont importants car ils permettent de supposer que non seulement le remodelage est une conséquence de l'inflammation tel que couramment proposé (An *et al.* 2007; Durrani *et al.* 2011), mais que la surrégulation des signaux menant à la prolifération est une réponse normale du muscle lisse. Cette surrégulation pourrait être utile dans certaines circonstances liées aux fonctions du muscle péribronchique, comme le bronchospasme en présence de substances irritantes. On peut supposer que lors d'inflammation, les mécanismes menant à l'hyperplasie musculaire se

mettent en place, mais ne mènent à une augmentation de masse que si l'inflammation est excessive et prolongée. La **Figure 24** illustre les effets de l'inflammation transitoire sur le muscle lisse de chevaux sains.

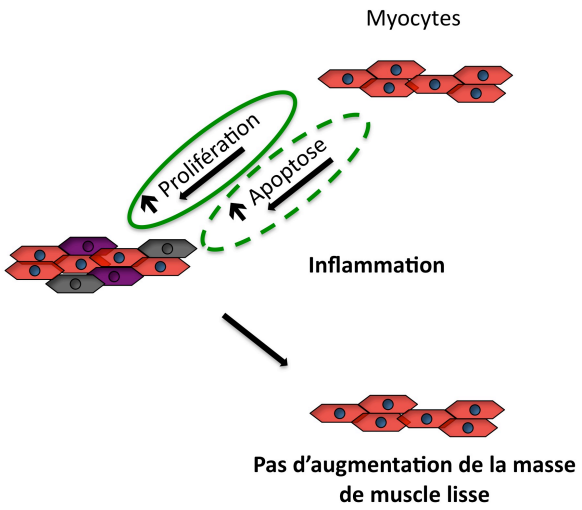


**Figure 24. Effets de l'inflammation transitoire sur le muscle lisse sain.**

Effet de l'inflammation sur muscle lisse de chevaux sains soumis à une inflammation pulmonaire secondaire à une exposition antigénique. L'inflammation transitoire résulte en une augmentation des marqueurs de prolifération (myocytes violets) sans résulter en une augmentation de masse, parce qu'elle est accompagnée d'une augmentation de l'apoptose (myocytes gris).

Un troisième aspect démontré dans l'étude I est que l'augmentation de masse semble due à la fois à de l'hyperplasie et à de l'hypertrophie des myocytes. L'augmentation de muscle lisse observée chez les chevaux atteints du souffle s'accompagne d'une augmentation du nombre de myocytes (hyperplasie), mais dans une proportion moindre que l'augmentation de masse, ce qui laisse supposer une augmentation concurrente de la taille des myocytes ou de la matrice extracellulaire les entourant (voir **Figure 4** et **Figure 25**).

Un certain degré d'hypertrophie semble effectivement contribuer à l'augmentation de masse, tel qu'indiqué par la diminution de la densité des noyaux (le nombre de noyaux par surface de muscle). L'hyperplasie et l'hypertrophie ont également été observées chez des sujets asthmatiques (Ebina *et al.* 1993; Regamey *et al.* 2008), alors que d'autres études n'ont démontré que l'un ou l'autre de ces phénomènes (Benayoun *et al.* 2003; Woodruff *et al.* 2004). Bien qu'il semble *a priori* logique que les deux processus (hyperplasie et hypertrophie) puissent coexister chez un même sujet, les études sur des myocytes en culture semblent plutôt montrer qu'il s'agit de phénomènes mutuellement exclusifs. En effet, le phénotype prolifératif / hyperplasie semble en opposition avec le phénotype hypercontractile / hypertrophique (Hirota *et al.* 2009). Ceci pourrait toutefois être vrai pour une cellule donnée qui doit consacrer ses ressources soit à se diviser, soit à se contracter et s'hypertrophier, mais pas pour un faisceau musculaire composé de nombreuses cellules, comme nos résultats le suggèrent.

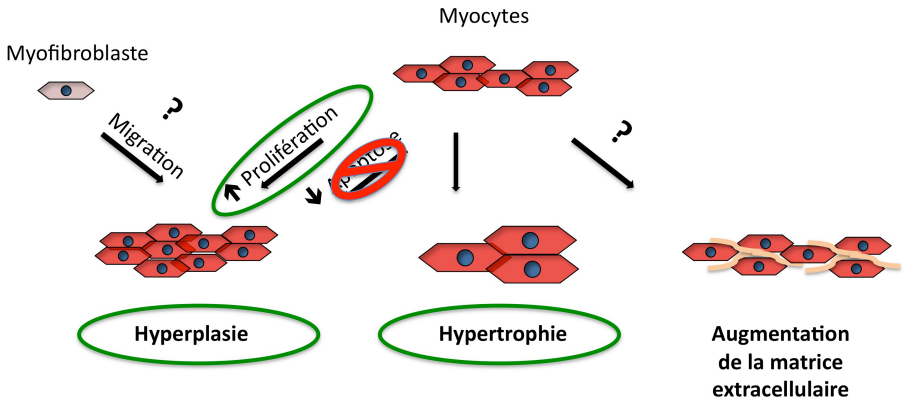


**Figure 25. Synthèse de l'étude 1.**

Résumé des conclusions de l'étude I sur les mécanismes menant à l'augmentation du muscle lisse chez les chevaux atteints du souffle.

*Les prélèvements biopsies endobronchiques ne permettent pas de mettre en évidence le remodelage musculaire chez les chevaux atteints du souffle*.

Un des objectifs de l'étude I visait à comparer, chez les mêmes animaux, le remodelage du muscle lisse observé via les biopsies obtenues par thoracoscopie au remodelage observé via les biopsies endobronchiques. Une bonne corrélation entre les deux aurait permis d'utiliser les biopsies endobronchiques pour des études subséquentes. Ces biopsies ont l'avantage d'être beaucoup plus faciles à obtenir, elles ne nécessitent pas de procédure chirurgicale ni de repos postopératoire et sont associées à très peu de complications. Elles sont cependant beaucoup plus petites que les biopsies obtenues par thoracoscopie et ne permettent pas une analyse en pleine épaisseur de la paroi des voies respiratoires. Dans l'étude I, la quantité de muscle lisse observée dans les biopsies endobronchiques était semblable chez les chevaux sains et les chevaux atteints du souffle asymptomatiques. Suite à l'exposition antigénique, le pourcentage de muscle est resté stable chez les chevaux sains mais il a *diminué* chez les chevaux atteints du souffle (**Figure 19A**). Ces résultats contrastent avec ceux d'études effectuées chez l'humain qui ont démontré, avec ce type de biopsies, une augmentation du muscle lisse chez les sujets asthmatiques (Benayoun *et al.* 2003; Kaminska *et al.* 2009; Pepe *et al.* 2005; Tillie-Leblond *et al.* 2008). Seules quelques études n'ont pas démontré de différences entre les sujets asthmatiques et les sujets sains (Labonte *et al.* 2008), ou n'ont observé de différence que chez les sujets sévèrement atteints (Benayoun *et al.* 2003). L'absence de différence entre les chevaux sains et malades en l'absence d'exposition antigénique pourrait s'expliquer soit par une technique d'échantillonnage inadéquate, soit par une absence de remodelage musculaire dans les voies centrales. Cette dernière explication faiblit à la lumière d'une évaluation détaillée des résultats de Herszberg et collègues. En effet, en réanalysant uniquement les voies respiratoires de calibre équivalant au calibre des voies qui peuvent être atteintes par bronchoscopie, on note que l'augmentation du muscle lisse chez les chevaux atteints du souffle est également présente dans les voies plus centrales (analyse des données originales de (Herszberg *et al.* 2006)). La

diminution du muscle suite à l'exposition antigénique peut quant à elle s'expliquer par une variation de la quantité et de la qualité des tissus prélevés. En effet, un épaississement des carinae secondaires était clairement observé par endoscopie lors des prélèvements biopsiques chez les chevaux symptomatiques. Il est possible que cet épaississement réduise la profondeur des biopsies. Il est aussi possible que l'élongation des voies respiratoires, décrite lors d'hyperinflation (Sasaki *et al.* 1996), fasse augmenter la distance entre l'épithélium et le muscle. Ces phénomènes ont comme conséquence de faire diminuer le pourcentage de tissu biopsié occupé du muscle, indépendamment de la masse totale de celui-ci. Ces artéfacts affectent peut-être davantage les biopsies endobronchiques faites chez les chevaux parce que les pinces utilisées sont plus petites relativement à la taille des carinae. De plus, chez l'humain, les biopsies sont rarement faites pendant une crise d'asthme. L'administration d'un bronchodilatateur pourrait atténuer l'effet de la bronchoconstriction sur la qualité des biopsies et sera considéré dans le futur. Une autre possibilité pouvant expliquer la diminution du muscle lisse est celle avancée par Kelly et collègues (Kelly *et al.* 2010). Dans cette étude, une diminution du muscle lisse a été observée dans des biopsies endobronchiques faites chez des patients asthmatiques 24 heures après une stimulation antigénique. Les auteurs suggèrent que cette diminution soit due à une dédifférenciation des myocytes en myofibroblastes. Les changements à court terme ainsi que la présence de myofibroblastes n'ont pas été étudiés ici et il n'est donc pas possible de commenter sur la présence ou l'absence de ce phénomène chez les chevaux atteints du souffle. Finalement, même s'il est difficile d'estimer la masse de muscle lisse présente autour d'une voie respiratoire à partir d'une biopsie de carina, il reste que ce type de biopsie permet de prélever suffisamment de muscle pour étudier la présence ou l'absence de certains marqueurs cellulaires ou pour effectuer des études d'expression génique. Elles ont en outre permis de mettre en évidence l'augmentation de la prolifération des myocytes dans les voies respiratoires centrales lors d'exposition antigénique (**Figure 19C** et **D**).

*Le remodelage musculaire chronique est partiellement réversible.*

L'aspect le plus important de l'étude II est d'avoir démontré que le remodelage chronique chez des animaux adultes est partiellement réversible. Ceci est nouveau et unique tant dans la littérature sur le souffle que celle sur l'asthme. Certaines études sur des sujets asthmatiques adultes ont déjà suggéré la possibilité d'une telle réversibilité, mais sans avoir pu observer l'évolution du remodelage dans le temps (Sumi *et al.* 2007) ou comparer avec des sujets sains (Bergeron *et al.* 2005). De plus, bien que des études sur les rongeurs aient montré un effet bénéfique des anti-inflammatoires sur le remodelage lorsqu'administrés précocement (Johnson *et al.* 2008; Siddiqui *et al.* 2010), ceci ne signifie pas pour autant que le remodelage chronique et stable observé chez des animaux malades depuis plusieurs années soit également réversible. Les chevaux étudiés dans les études I et II présentaient tous des signes cliniques depuis quatre à dix ans, période au cours de laquelle ils avaient eu plusieurs épisodes de bronchoconstriction et d'inflammation nécessitant une intervention. Ceci laisse penser que le remodelage musculaire était présent depuis plusieurs années, et qu'il avait possiblement atteint un plateau dans sa progression, tel que discuté précédemment. L'implication majeure de cette partie de l'étude II pour le traitement du souffle et de l'asthme est qu'une intervention, même tardive, chez des sujets atteints depuis de nombreuses années peut être bénéfique non seulement pour contrôler les signes cliniques, mais aussi pour limiter les effets néfastes du remodelage. Un effet bénéfique des corticostéroïdes sur le déclin de la fonction respiratoire a d'ailleurs été observé chez des patients asthmatiques (O'Byrne *et al.* 2006; Pauwels *et al.* 2003) et l'étude II supporte l'hypothèse proposée par ces auteurs, à savoir que cet effet bénéfique est dû à une diminution du remodelage.

*Une portion du remodelage musculaire chronique semble réfractaire à une intervention et pourrait être permanente.*

Bien qu'une diminution significative de la masse de muscle lisse ait été observée, la masse présente à la fin de l'étude était toujours plus de deux fois celle des chevaux sains de

l'étude I et de ceux de l'étude de Herszberg et collègues. Il est donc possible qu'une réversibilité complète ne puisse se faire que sur une plus longue période et que la durée de l'étude II (1 an) n'ait pas permis d'observer une diminution maximale. Toutefois, deux aspects des résultats obtenus suggèrent qu'une portion du remodelage est irréversible. Premièrement la diminution observée est modeste et similaire peu importe l'intervention (corticostéroïdes administrés par inhalation ou réduction de l'exposition antigénique). Deuxièmement, l'intervention qui a entraîné une diminution la plus *rapide* (corticostéroïdes par inhalation) n'a pas mené à une diminution totale plus *importante*. Dans ce groupe, la masse de muscle lisse est restée stable pendant les six derniers mois de l'étude, alors que les chevaux étaient à la fois dehors (faible exposition antigénique) et sous corticothérapie. Ceci suggère que le remodelage est en partie irréversible. Les études effectuées ici ne permettent pas d'expliquer pourquoi le remodelage serait en partie irréversible mais les hypothèses suivantes peuvent être émises. Il est possible que d'autres aspects du remodelage, comme le dépôt de collagène par exemple, entraîne un changement de structure tel qu'un retour à une quantité normale de muscle serait néfaste pour le maintien de la structure des voies respiratoires. Cette idée rejoint l'hypothèse qu'une partie du remodelage serait une adaptation bénéfique pour résister aux changements de force transmise par les tissus environnants (Bergeron and Boulet 2006; Palmans *et al.* 2000). Il est aussi possible que, malgré les interventions sur l'environnement et avec un traitement anti-inflammatoire, un certain degré d'inflammation pulmonaire ait persisté et entretenu les modifications tissulaires. Cette inflammation persistance est hypothétique car elle n'a pu être mise en évidence dans les lavages bronchoalvéolaires ou les biopsies pulmonaires. L'implication de cette irréversibilité partielle possible du remodelage musculaire est que, même si une intervention visant le remodelage peut être utile, une intervention plus précoce ou ciblant plusieurs aspects du remodelage serait plus bénéfique pour les patients.

*Les corticostéroïdes et les modifications environnementales ont mené à une même diminution du muscle lisse, mais en deux fois moins de temps avec les corticostéroïdes.*

La première conclusion qui vient à l'esprit devant ce constat est que le contrôle de l'inflammation, peu importe le moyen utilisé, est le déterminant principal de la réversibilité du remodelage. Toutefois, une analyse plus poussée des résultats semble indiquer que les mécanismes impliqués pourraient être plus complexes et pourraient même remettre en question l'hypothèse voulant que le remodelage, vraisemblablement causé par l'inflammation chronique, serait réversible via un contrôle de cette inflammation (Durrani *et al.* 2011). On note notamment que dans le groupe sous corticostéroïdes, le muscle lisse diminue rapidement dans les six premiers mois, alors que l'inflammation persiste dans le lavage bronchoalvéolaire durant cette période. Une dissociation entre l'amélioration de l'inflammation dans le lavage bronchoalvéolaire et la diminution du remodelage est aussi observée dans le groupe traité uniquement par une diminution de l'exposition antigénique. Dans ce groupe, alors que le lavage bronchoalvéolaire se normalise plus rapidement que dans le groupe sous corticothérapie, l'amélioration du remodelage musculaire est plus lente. Récemment Siddiqui et collègues (Siddiqui *et al.* 2010), travaillant sur un modèle d'asthme chez le rat, ont également observé un effet positif des corticostéroïdes sur le remodelage du muscle lisse, sans effet sur la cytologie des lavages bronchoalvéolaires. Ceci pourrait refléter un effet direct des corticostéroïdes sur la prolifération des myocytes (Burgess *et al.* 2008; Roth *et al.* 2004; Schramm *et al.* 1996; Young *et al.* 1995) ou un effet indirect via la diminution du bronchospasme (par leurs effets sur la contractilité discutés dans « Effets des corticostéroïdes sur le muscle lisse »), ce dernier pouvant en soi contribuer au remodelage (Grainge *et al.* 2011). Une explication plus simple serait que la cytologie des lavages bronchoalvéolaires ne reflète pas l'inflammation dans le microenvironnement des myocytes péribronchiques. Pour cette raison, l'inflammation tissulaire dans les biopsies de l'étude II a été évaluée, mais un meilleur contrôle dans le groupe sous corticothérapie n'a pas été mis en évidence. Cette évaluation histologique a seulement permis de mettre en évidence une diminution de l'inflammation au cours du temps dans le groupe traité par une diminution de l'exposition antigénique (**Figure 23**). L'analyse des cytokines dans le lavage bronchoalvéolaire, l'évaluation de l'inflammation systémique et la recherche de cellules

inflammatoires immédiatement adjacentes au muscle lisse pourraient permettre de préciser si l'action principale des corticostéroïdes sur le remodelage s'exerce effectivement via leurs effets anti-inflammatoires. Il semble également que le degré d'activation des neutrophiles soit augmenté dans le souffle (résumé par (Leclere *et al.* 2011) et l'asthme (Macdowell and Peters 2007), et il pourrait être sous régulé par les corticostéroïdes (Gin and Kay 1985).

*Autres contributions à l'avancement des connaissances*

D'autres contributions à l'avancement des connaissances proviennent d'études effectuées en parallèle des études I et II présentées ici. Les deux plus importantes portent d'une part sur l'analyse de l'expression génique du tissu pulmonaire et d'autre part, sur les effets des corticostéroïdes administrés par inhalation sur la fonction immunitaire. L'expression génique du tissu pulmonaire de chevaux atteints du souffle a été investiguée en utilisant une portion des tissus prélevés dans l'étude I. Même si la majorité des manipulations en laboratoire n'ont pas été effectuées par l'auteur de cette thèse, cette étude a influencé les protocoles et les techniques de biopsies utilisés pour ces travaux, ceci afin de respecter les contraintes liées à l'analyse de l'ARN messager. Ainsi, il a fallu limiter la manipulation des tissus et les substances administrées aux animaux lors soins préopératoires et durant les thoracoscopies. La technique d'hybridation suppressive soustractive (SSH) a permis d'identifier des gènes surexprimés chez les chevaux atteints du souffle en crise, par rapport à des chevaux sains et des chevaux atteints mais asymptomatiques. La technique consiste à « soustraire » les gènes exprimés chez les chevaux sains et les chevaux atteints mais asymptomatiques, des gènes exprimés chez les chevaux atteints en période d'exacerbation. Des 950 gènes surexprimés, 224 ont été séquencés. Pour s'assurer que la soustraction ait été efficace, certaines de ces séquences codant pour des gènes connus ont été quantifiées par PCR, qui a confirmé le pattern d'expression pour 15 des 22 gènes testés. Certains gènes identifiés sont intéressants pour leur rôle dans l'inflammation pulmonaire tandis que d'autres ont possiblement un rôle à jouer dans le remodelage. Une quinzaine de gènes sont associés à la contraction musculaire ou au remodelage du muscle lisse, comme la

calcineurine et le récepteur à l'endothéline A (Annexe 4) (Lavoie *et al.* 2011). Dans le contexte du remodelage du muscle lisse péribronchique, ces données pourront servir d'assises pour l'étude de l'expression génique spécifique au muscle microdisséqué. La mise au point de la technique de microdissection au laser (**Figure 26**) et l'optimisation de la préservation d'une qualité adéquate d'ARN ont été réalisées. Les analyses du muscle lisse péribronchique du poumon périphérique prélevé dans l'étude I sont actuellement en cours.

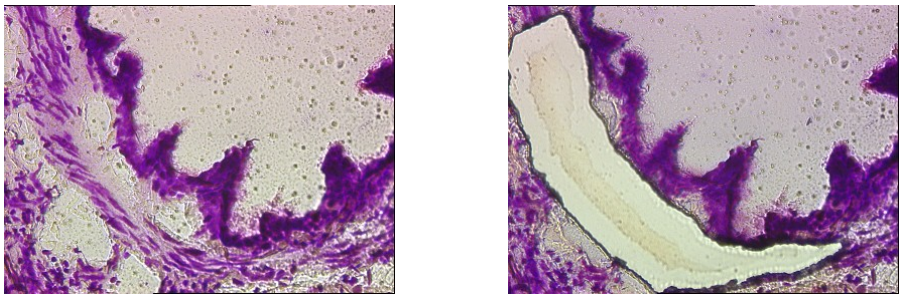

**Figure 26. Microdissection au laser de muscle lisse péribronchique.**

Coupe de tissu pulmonaire périphérique prélevé dans l'étude I avant et après microdissection au laser du muscle lisse péribronchique.

La seconde étude d'importance, faite en parallèle de l'étude II, porte sur les effets des corticostéroïdes administrés par inhalation sur la réponse immunitaire. Un des buts de l'administration des corticostéroïdes par cette voie plutôt par voie orale ou intramusculaire est de minimiser les effets secondaires systémiques, tout en atteignant des concentrations thérapeutiques optimales au niveau pulmonaire. Cet avantage a été démontré chez d'autres espèces mais les effets des corticostéroïdes administrés par inhalation sur le système immunitaire des chevaux n'ont jamais été évalués en profondeur. La conclusion de cette étude est que l'administration de fluticasone par inhalation pendant un an à dose thérapeutique n'a pas d'effet détectable sur l'immunité innée et acquise (humorale et à

médiation cellulaire), et ne devrait donc pas compromettre la réponse vaccinale ni la défense contre les pathogènes des chevaux adultes (Dauvillier *et al.* 2011).

# Conclusion et perspectives

L'hypothèse selon laquelle les chevaux atteints du souffle ont plus de muscle lisse péribronchique que les chevaux sains d'âge similaire, gardés dans les mêmes conditions environnementales et l'hypothèse selon laquelle la prolifération des myocytes contribue à l'augmentation de la masse de muscle lisse ont été vérifiées par ces études (hypothèses 1 et 3 de l'étude I). Une stimulation antigénique relativement courte n'accentue toutefois pas la masse de muscle lisse de ces chevaux affectés de façon chronique (hypothèse 2 de l'étude I), possiblement parce le remodelage atteint un plateau (pour le poumon périphérique) ou parce que le muscle ne peut être quantifié adéquatement dans les biopsies endobronchiques chez les chevaux (hypothèse 4 de l'étude I). Le remodelage chronique du muscle lisse respiratoire est partiellement réversible avec l'administration de corticostéroïdes par inhalation ou une modification environnementale prolongée (hypothèse 1 l'étude II) mais la combinaison de corticostéroïdes inhalés et de modifications environnementales n'accentue pas la diminution du remodelage musculaire (hypothèse 2 l'étude II). La réversibilité du remodelage par une diminution de la prolifération des myocytes n'a pas été démontrée mais ne peut être exclue étant donnée la tendance vers la diminution de la prolifération des myocytes observée lors de la diminution la plus rapide du muscle lisse (entre 0 et 6 mois) (hypothèse 3 l'étude II).

La **Figure 27** représente de façon schématique le résumé des travaux présentés ici, en intégrant les connaissances actuelles et en proposant une hypothèse de travail. Selon cette hypothèse, l'augmentation de la masse de muscle lisse a principalement lieu lors des premiers épisodes d'inflammation causée par l'exposition antigéniques des animaux susceptibles. Parce que des mécanismes existent pour empêcher une hyperplasie et hypertrophie incontrôlées, le remodelage atteint un plateau et est maintenu dans cet état de stabilité dynamique par les épisodes d'inflammation. Une intervention soutenue permet de diminuer la masse, possiblement en affectant le taux de prolifération des myocytes mais une inflammation sous clinique pulmonaire ou systémique, ou encore d'autres aspects du remodelage, empêche un retour à la normale.

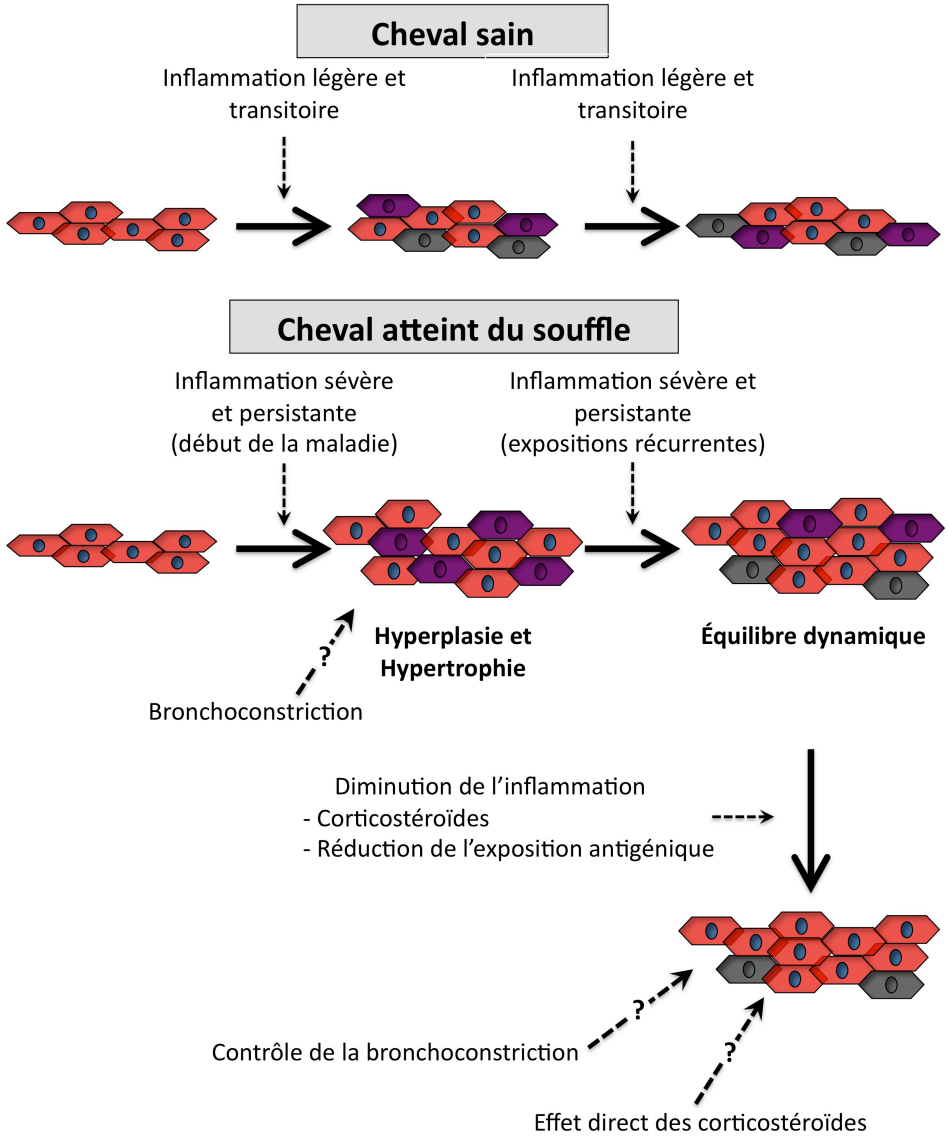


**Figure 27. Mécanismes proposés pour expliquer le remodelage et sa réversibilité.**

Proposition des mécanismes impliqués dans le remodelage du muscle lisse dans les maladies inflammatoires chroniques. Chez les chevaux atteints du souffle, l'inflammation persistante entraîne une augmentation de la masse de muscle lisse via une prolifération *in situ* (myocytes violets) et de l'hypertrophie. Avec le temps, les expositions antigéniques subséquentes maintiennent un turnover élevé (prolifération et apoptose élevées (myocytes

gris)) mais en maintenant une masse totale stable. Avec une diminution de l'inflammation, les signaux pro-prolifératifs diminuent, faisant diminuer la masse de muscle lisse. Chez les chevaux sains, l'inflammation légère, « normale », n'entraîne pas d'augmentation de masse, même si les myocytes ont la possibilité de répondre aux signaux inflammatoires. Les possibles effets directs des corticostéroïdes et de la bronchoconstriction sur le remodelage sont également illustrés.

## Perspectives

Les premières étapes des études à venir seront de mesurer l'effet d'une intervention au niveau des autres aspects du remodelage (épithélium, matrice extracellulaire). Ceci pourra être fait sur les biopsies récoltées dans le cadre de ces études et certains aspects sont déjà en cours d'investigation. Comme on suspecte que l'inflammation résiduelle joue un rôle dans l'amélioration limitée du remodelage du muscle lisse observée dans l'étude II, des moyens plus sensibles et plus spécifiques pour mesurer l'inflammation péribronchique devraient être développés. Ceci pourrait être fait entre autre via des marqueurs d'inflammation tels que les protéines de la phase aiguë dans la circulation sanguine (résultats préliminaires présentés récemment (Lavoie-Lamoureux *et al.* 2011)). Étant donné qu'il est possible que les corticostéroïdes agissent rapidement sur la masse de muscle, il serait intéressant d'explorer les changements au niveau du muscle lisse peu après le début de la corticothérapie. L'approche par thoracoscopie se prête difficilement à des prises de biopsies à très courts intervalles, surtout si un des focus d'étude est l'inflammation. Toutefois, certaines techniques d'imagerie, comme l'échographie endobronchique à haute résolution, pourraient permettre de quantifier le remodelage musculaire alors que l'immunomarquage et l'étude de l'expression génique pourraient être faites sur des biopsies endobronchiques effectuées en parallèle.

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# Annexe 1

**Heaves, an asthma-like disease of horses**

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* M.L. et A.L.-L. ont participé de façon égale à la rédaction de cet article

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Sommaire

Cette publication est une revue des connaissances sur le souffle en tant que modèle animal d'asthme. Les similitudes et les différences entre ces deux maladies et les autres modèles d'asthme sont présentées, pour un auditoire médical non vétérinaire. L'étiologie, la réponse immunitaire, la dysfonction respiratoire, l'inflammation pulmonaire et systémique, le remodelage des voies respiratoires et les traitements du souffle sont détaillés au meilleur des connaissances actuelles.

**Contribution**
A.L.L. et moi-même avons contribué de façon équivalente à la revue de la littérature et la rédaction de cet article comme co-premiers auteurs.

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# Heaves, an asthma-like disease of horses

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## ABSTRACT

**Animal models have been developed to investigate specific components of asthmatic airway inflammation, hyper-responsiveness or remodelling. However, all of these aspects are rarely observed in the same animal. Heaves is a naturally occurring disease of horses that combines these features. It is characterized by stable dust-induced inflammation, bronchospasm and remodelling. The evaluation of horses during well-controlled natural antigen exposure and avoidance in experimental settings allows the study of disease mechanisms in the asymptomatic and symptomatic stages, an approach rarely feasible in humans. Also, the disease can be followed over several years to observe the cumulative effect of repeated episodes of clinical exacerbation or to evaluate long-term treatment, contrasting most murine asthma models. This model has shown complex gene and environment interactions, the involvement of both innate and adaptive responses to inflammation, and the contribution of bronchospasm and tissue remodelling to airway obstruction, all occurring in a natural setting. Similarities with the human asthmatic airways are well described and the model is currently being used to evaluate airway remodelling and its reversibility in ways that are not possible in people for ethical reasons. Tools including antibodies, recombinant proteins or gene arrays, as well as methods for sampling tissues and assessing lung function in the horse are constantly evolving to facilitate the study of this animal model. Research perspectives that can be relevant to asthma include the role of neutrophils in airway inflammation and their response to corticosteroids, systemic response to pulmonary inflammation, and maintaining athletic capacities with early intervention.**

**Key words:** animal model, asthma, inflammation, remodelling.

## INTRODUCTION AND DEFINITION

In the past decades, much has been learned on the pathophysiology of asthma from the study of human patients and animal models. Gene and environment interactions, which vary based on the type and duration of antigen exposure as well as other triggering events, have been highlighted. Many of these complex events are difficult to model in rodents. Equine heaves is a spontaneous occurring asthma-like condition affecting a domestic animal species. In this review, we will describe the characteristic features of heaves and highlight novel findings particularly relevant to human asthma.

Heaves is estimated to affect approximately 10–20%[1] of adult horses in the northern hemisphere and other temperate climates. Descriptions of horses with a respiratory disease similar to what is now known as heaves can be found as far as in ancient Greece. In 1893, Bouley described as 'nervous heaves' ('pousse' in French lay term), a condition of horses associated with marked breathing difficulty, but without obvious macroscopic pathological lung lesions.[2] Perhaps in the first 'scientific report' of the disease, Lowel[3] captured the main clinical features of the disease and recognized it as analogous to human asthma.

Similarly to asthma, heaves is a chronic disorder of the airways, which is characterized by variable and recurring airflow obstruction, bronchial hyperresponsiveness and airway inflammation. During disease exacerbation, horses present increased respiratory efforts at rest, coughing and exercise intolerance. Clinical signs are triggered or exacerbated by inhalation of dust particles present in the stables, especially those associated with hay feeding. For these reasons, heaves is regarded as a disease of domestication. Experimentally, stabling susceptible

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horses in the presence of hay and straw, both sources of antigen-rich organic dust, exacerbates heaves. Pollens and various other antigens found in high levels outdoors in the spring and summer may also be implicated.[4–6] The condition is then identified as 'summer pasture-associated obstructive pulmonary disease (SPAOPD)'. Specific reference to SPAOPD will only be made when of interest to this review.

When stabled, heaves-susceptible horses develop a marked and persistent airway inflammation believed to be a consequence of aberrant innate and adaptive immunity responses, as well as an underlying genetic component. During exacerbation, the major cause of airflow limitation is bronchospasm, as indicated by the rapid and significant (60–70%) improvement in lung function observed following the administration of bronchodilators.[7–9] The relative contribution of mucus, inflammatory cells and airway wall remodelling to airway obstruction during disease exacerbation and to the residual function impairment measured in asymptomatic horses[10] remains speculative.

## NOMENCLATURE IN THE VETERINARIAN LITERATURE

Heaves and Recurrent Airway Obstruction (RAO) are currently used indistinctly in the veterinary literature.[11] The terms 'chronic obstructive pulmonary disease' and 'chronic bronchitis', mainly used in the 1980s and 1990s literature, have been discarded because the human counterparts of these syndromes have different causes, clinical features and pathophysiology. Similarly, 'equine emphysema' is improper as air trapping rather than alveoli wall disruption is responsible for the lung hyperinflation observed in severely affected horses.[12,13]

While asthma describes a clinical syndrome of variable severity, the condition affecting horses with milder respiratory impairment and airway inflammation is referred to as 'inflammatory airway disease (IAD)'. Whereas airway neutrophilia predominates in heaves, neutrophils, metachromatic cells and eosinophils may be present in increased number in broncho alveolar lavage fluid (BALF) of horses with IAD.[14] Horses with airway eosinophilia tend to be younger, and those with neutrophilia older than controls.[15] These findings are reminiscent of the heterogeneous asthma phenotypes.[16] It is currently unknown which horses with IAD eventually progress to the severe clinical signs observed in heaves.

## AETIOLOGY AND PATHOPHYSIOLOGY

### What makes a horse susceptible to heaves

It is generally estimated that 10–20% of adult horses living in cold and temperate climates are affected by heaves, although epidemiological reports are few. Disease prevalence is approximately 14% (95% CI: 10.7–17.4%) in horses aged 5 years or more in the UK, as estimated using an owner-answered, validated questionnaire.[1,17] A strong genetic determinant has been observed in some families of horses with chronic airway inflammation and the prevalence of disease in offsprings born from two affected parents can reach approximately 38–48%, while those born from one affected parent are less at risk (approximately 6–17%).[18] No breed or gender predisposition have been identified.[1,18–20]

As human asthma, heaves likely results from the interactions between multiple genes and environmental factors.[21] Polymorphisms were identified in a region harbouring the *IL4RA* locus on the equine chromosome 13 (ECA13q13)[22] and were associated with heaves in one of two high prevalence families.[23] Similarly, *IL4RA* polymorphisms are associated with phenotypes of asthma and atopy in humans as well as changes in the IL-4 receptor function.[24,25] Recent studies have found an association between heaves and resistance to parasitic infections in two unrelated high prevalence families.[26,27] This association has also been reported in allergic people.[28] The genetic determinants of allergic diseases may therefore confer resistance to certain parasitic infections, particularly Th2-related genes (including the IL-4Rα gene), which are directly involved in the host defence against these pathogens.[28] There is little information on early life events that can be linked to the development of heaves. However, in a questionnaire-based survey of 361 owners, Hotchkiss identified early life exposure to hay and reported respiratory infection before the age of 5 years as risk factors for horses with heaves.[1] These findings are also reminiscent of the increased risk of developing asthma in children who experienced viral infection during their first years of life.[29]

### What makes a susceptible horse symptomatic

Exacerbation of heaves is clearly linked to environmental dust exposure. With only a relatively small subset of horses to develop heaves, the weight of evidence suggests that the disease is the result of allergic sensitization to airborne organic particles present in stables. However, the exact antigens responsible for the onset of clinical signs in susceptible animals remain uncertain. Stable dust contain more than 50 types of moulds, mites, bacterial endotoxins and inorganic compounds that may contribute to the disease.[11] Other particles present in the horse environment may also contribute to bronchoconstriction, either because the airways are intrinsically hyper-reactive in heaves or because they become hyper-reactive when inflamed. Air pollution is another possible contributing factor, as urbanized environment has been associated with an increased risk for developing heaves.[1] However, this could be explained by other factors, such as increased antigen load exposure resulting from limited pasture access in these areas.


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## Specific response to antigens

Inhalation of soluble mould extracts of *Aspergillus fumigatus* and *Faenia rectivirgula*, but not extracts of *Thermoactinomyces vulgaris*[30] or hay pollen,[3] leads to airway neutrophilia and obstruction in heaves-susceptible horses but not in healthy controls. These observations suggest that sensitization and allergic responses to antigens from these moulds are involved in heaves pathogenesis. However, studies have failed to show disease-specific skin reactivity to barn allergens,[3,31] although higher frequency of positive skin reactions is found in affected horses compared with unaffected horses.[32–34] Higher concentrations or greater frequency of detection of antibodies (IgG and IgE) specific to *A. fumigatus* or *Alternaria alternate* crude extracts or recombinant antigens, respectively, were found in serum samples of heaves-affected compared with control horses in some,[35–39] but not in all studies.[40,41] Nevertheless, the overlapping ranges in specific antibody concentration or skin prick test positivity between normal and heaves-affected horses preclude their use as tools for the diagnosis of heaves. Of note, horses were shown to generally develop serum IgG and IgE antibodies towards common mould antigens in a manner that highly depends on environment, genetic background, gender and age.[37,42] In BALF, significantly higher concentrations of specific IgE, IgG and IgA antibodies against *A.fumigatus* and *Micropolyspora faeni* were found in heaves-affected horses compared with controls, suggesting local production of specific antibodies in affected animals.[40,41] This may be comparable to a recently defined immunological process called 'entopy', which describes a 'local allergy in non-atopic individuals (. . .) which may or may not involve locally produced IgE'[43] and/or a local Th2-driven inflammation[44] occurring in asthmatic patients without skin prick test positivity or serum antigen-specific IgEs (intrinsic asthma).[45]

An immediate allergic response is not typically associated with heaves exacerbation and an early phase (10–20 min) histamine release and bronchoconstriction response to inhaled dust does not occur in diseased horses.[46] A significant histamine release and bronchoconstriction is however reported in healthy controls, suggesting that the early phase response may contribute to reduce the amounts of antigens reaching the peripheral airways and therefore be protective.[46] Nevertheless, pulmonary mast cells from heaves-affected horses have a greater *in vitro* histamine release in response to mould extracts (*A. fumigatus*, *M. feani*, *A. tenius*)[47] and greater histamine concentrations are detected in pulmonary epithelial lining fluid from these horses compared with controls 5 h, but not 30 min, after natural antigenic challenge.[48] In addition, tryptase$^{+49}$ or chymase$^{+50}$ mast cells are increased in the epithelium and peribronchial space, respectively, of heaves-affected compared with healthy horses and tryptase is higher in BALF of diseased animals[49] from 1 to 5 days after exposure to a natural antigenic challenge. Taken together, these observations suggest that a typical Type I hypersensitivity response characterized by both early and late phase responses does not define heaves pathology. Rather, a specific IgE/mast cells-mediated *late phase* inflammation and bronchoconstriction possibly contributes to the disease, although the role of IgE in the pathogenesis of heaves remains controversial.[51] The delayed neutrophilic inflammation following dust inhalation is reminiscent of the type III hypersensitivity observed in allergic pneumonitis (farmer's lung disease). However, alveolar inflammation and granuloma formation combined with fever are not features of heaves pathology in contrast to allergic pneumonitis.[52] Furthermore, precipitins that are used as markers of this disease are not specifically found in the serum or lungs of horses with heaves.[53]

### *T cell-derived cytokines associated with heaves (Th1/Th2/Th17)*

The cytokine profile associated with heaves exacerbation has been broadly investigated. In agreement with the hypothesis that a local allergy-like response is involved in heaves pathology, a higher frequency of lymphocytes expressing Th2-type cytokines (IL-4, IL-5) and a decreased expression of a Th1-type cytokine (INFγ) was found in BALF of affected horses using *in situ* hybridization.[54,55] Also, in another study INFγ was found to be decreased in BALF CD4+ lymphocytes.[56] Using a model of ovalbumin-sensitized and challenged ponies, Bowles and colleagues showed the concomitant development of airway neutrophilia with an increased expression of Th2-type cytokine mRNAs in BALF cells (IL-4, IL-5, IL-13) from ovalbumin-sensitized compared with unsensitized controls, supporting a contribution of these cytokines in driving airway neutrophilia.[57] Contrasting results were found by other teams using PCR on total BALF cells, who found predominant expression of INFγ alone[58,59] or combined with Th2 cytokines (IL-4 or IL-13),[60,61] during the exacerbation phase of the disease. Total TGF-$\beta_1$ concentration is not changed in BALF from symptomatic heaves-affected horses although the distinction between active and inactive TGF-$\beta_1$ was not investigated.[62] Finally, increased IL-17A expression in BALF cells from heaves-affected horses during exacerbation has been reported.[63,64]

Several factors may contribute to these conflicting findings. The genetic background of the host could influence its immune response, as is the nature and duration (acute vs chronic) of the natural antigenic exposure. Indeed in asthma, Th1, Th2 and Th17 responses have been reported with different exacerbation triggering factors (virus, allergens) or severity,[65–68] and lymphocytes producing a mixed Th2/Th17 cytokine profile have been recently identified in asthmatic patients.[69] Nevertheless, the local production of specific antibodies and the response of affected horses to inhalation challenge with specific allergens suggest that lymphocyte-driven (adaptive) immune response to allergens is involved in heaves pathophysiology.

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### Innate immune responses: contribution of non-specific factors

Inhalable endotoxin levels in conventional stables (hay and straw regiment, poorly ventilated) where susceptible horses develop heaves symptoms are 8–15 times higher than in low dust stables (well ventilated, hay pellets and shavings) or pasture where reversal of airway obstruction and inflammation is reached.[70,71] These respirable endotoxin levels (1.7 mg/m$^3$) are comparable to those found in certain house dust,[72] and cotton[73] or grain industries[74] linked to chronic airway diseases in humans. Healthy humans develop lung dysfunction following inhalation of 80 μg of endotoxin,[75] whereas the threshold response was found to be lower in asthmatics patients (20 μg). Similarly, it was shown that doses 10 times lower of endotoxin were sufficient enough to induce airway neutrophilia in horses with heaves compared with controls (20 vs 200 μg), and that only affected horses develop lung dysfunction with endotoxin inhalation alone.[76] The concentrations necessary to induce airflow limitation are however considerably greater than those found in stable dust, suggesting that it is not the main driving factor in heaves exacerbation. Synergistic interactions between allergens and non-specific agents may nonetheless be involved as endotoxin depletion experiments showed that it contributed significantly to airway neutrophilia and lung dysfunction in response to either hay dust suspension (HDS) or *A. fumigatus* extracts inhalation.[77,78] As expected with a strong response to endotoxin, NF-κB activity is higher in the airways and BALF cells of horses with heaves.[79,80] It was hypothesized that activated leukocytes secreting pro-inflammatory cytokines have an autocrine and paracrine effect on NF-κB activation and sustain inflammation of the airways. In addition, TLR4 gene expression was found to be increased in BALF cells of heaves-affected horses during organic-dust exposure,[64] and polymorphisms in TLR4 sequence has been reported in horses.[81] These observations suggest that innate mechanisms also contribute to heaves pathophysiology. Other agents present in stable dust (β-glucans, mites, moulds spores, noxious gas)[82,83] could also enhance immune responses in susceptible horses.

## LUNG FUNCTION

### Means of quantifying respiratory dysfunction in heaves-affected horses

Airway obstruction and its consequences on gas exchange can be measured by different methods in horses. Due to their quiet demeanour, most techniques can be performed on conscious, standing animals, and on multiple occasions, without generating stress artefacts. Conventional lung function measurement with the use of an esophageal catheter and a pneumotachograph (Fig. 1a) reveals increased pulmonary resistance and decreased dynamic compliance during exacerbation of heaves.[84–86] The former is caused mainly by bronchospasm, as shown

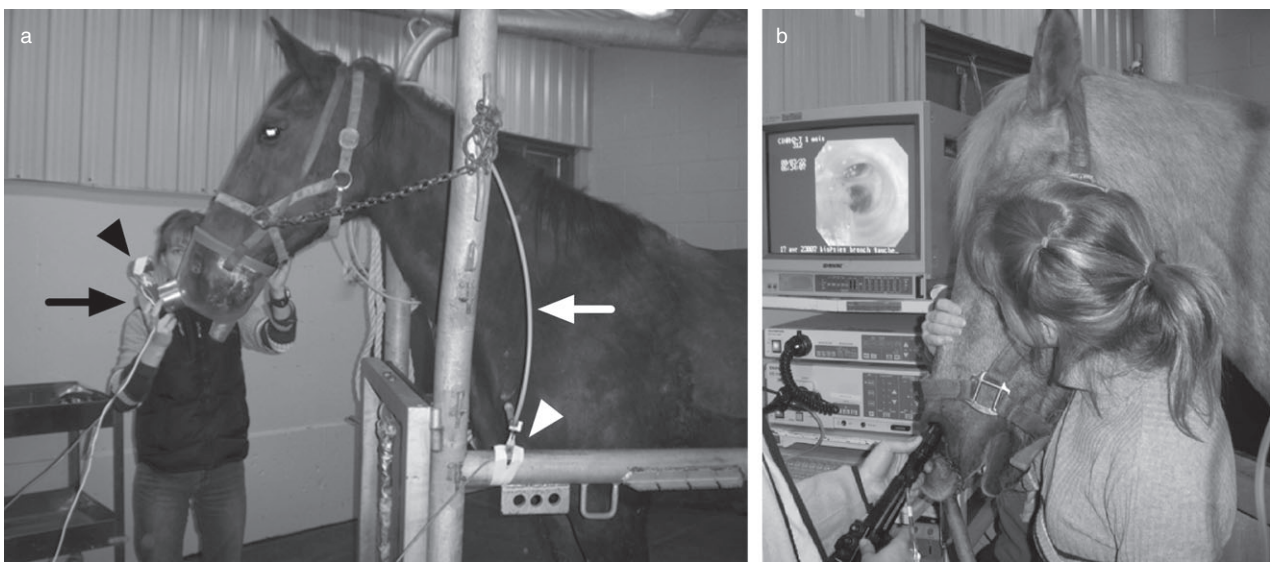


**Figure 1** (a) Conventional lung function testing in an unsedated heaves-affected horse. The esophageal catheter (white arrow) is placed through one nostril (on the right here), down to the caudal thoracic esophagus and connected to a pressure transducer (white arrowhead). A low resistance mask is placed over the horse's nose and flow rates are obtained from a heated pneumotachograph (black arrow) and an associated differential pressure transducer (black arrowhead). Signals are passed through a digital/analog converter to a computer equipped with a data acquisition and analysis software. Note that horses are obligate nasal breathers. (b) BAL performed on a standing, sedated horse with heaves.


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by a rapid decline in resistance after bronchodilator administration; resistance values commonly decrease by 60–70%[7–9] but remain above those observed on pasture or of healthy controls, suggesting residual airway obstruction by mucus and inflammatory cells, and possibly airway wall remodelling.[87] The decrease in dynamic compliance is less consistently responsive to bronchodilator administration, possibly because of peripheral airways obstruction, uneven distribution of ventilation and parenchymal remodelling.[7,8] Abnormal ventilation distribution has been shown by nitrogen washout,[88] helium-dilution measurements,[89] radiolabelled aerosolized technetium[90] and, indirectly, by volumetric capnography.[91] Ventilation/perfusion (V/Q) mismatch has also been shown to be most important during episodes of clinical exacerbation and to improve without disappearing following treatment.[92,93] Peak expiratory (mostly) and inspiratory flow,[94–96] the work of breathing,[86,97] and the variation in pleural pressure during tidal breathing increase markedly, which give rise to the marked costal excursion and prolonged abdominal contraction observed clinically.[94] Typically, tidal volume is maintained and minute ventilation increases due to a rise in respiratory rate.[94] Figure 2 illustrates the changes in resistance before and after natural antigenic exposure, and following the administration of corticosteroids and a bronchodilator.

Standard respiratory mechanic measurements have proven very useful in discriminating between heaves-affected and healthy controls during stabling and in assessing response to therapy. However, despite evidence of persistent mild respiratory dysfunction (AaDO2, V/Q mismatch,[92] lack of normal biphasic inspiration and expiration[96]) during clinical remission, pulmonary resistance and dynamic compliance, values are often similar to those of healthy subjects.[11] By imposing external forces to the respiratory system over a wide range of frequencies, forced oscillation techniques, at least in theory, can detect more subtle function impairment and help to localize the site of the anomaly (large airways, peripheral airways, parenchyma). In horses, forced oscillation techniques are less invasive than standard respiratory mechanics,[99] they correlate well with conventional lung function measurement during histamine bronchoprovocation,[100,101] and they can detect subclinical airway obstruction in heaves and IAD.[102,103] Whole body plethysmography is another non-invasive technique that has been used to evaluate function and response to therapy,[104] but inherent technical difficulties have limited its use. Volumetric capnography and inductance plethysmography portability may prove helpful for the assessment of airway function in field studies.[91,105]

Forced expiration technique is not widely used in horses, as it requires heavy sedation, nasotracheal intubation, mechanical ventilation and the application of a vacuum. However, it detects early onset of pulmonary dysfunction in heaves,[106,107] and persistent airway limitation during clinical remission, even after years of strict environmental control.[10]

### Non-specific airway hyper-responsiveness

Airway hyper-responsiveness (AHR) to non-specific agonists is also present in heaves and is increased when affected horses are exposed to organic dust,[108–111] even before changes in baseline lung function and clinical signs can be observed.[112] While many have shown that AHR subsides when horses are on pasture,[8,110,112–114] some have found that AHR can persists in asymptomatic horses with no exposure to hay.[101] This non-specific hyper-responsiveness could reflect persisting inflammation,[115] airway wall remodelling, intrinsic smooth muscle sensitivity/reactivity, or autonomic dysfunction.

### Gas exchange, ventilation/perfusion and epithelium integrity

Arterial hypoxemia, prolonged nitrogen washout, dead space ventilation and ventilation of high V/Q regions are present in heaves. Arterial blood gas is easily collected in horses and oxygen tension is decreased during exacerbation, however the degree of hypoxemia is poorly correlated with pulmonary mechanics.[116] Prolonged nitrogen washout is consistent with the severity of histological lung changes,[84,117] and bronchiolar epithelial hyperplasia is observed in regions with high V/Q and dead space ventilation.[93] Ventilation/perfusion heterogeneity improves but persists with treatment.[92] Ventilation/perfusion mismatch and alveolar hypoxia can lead to increased pulmonary vascular resistance and pulmonary arterial pressure,[93] and secondary cardiovascular changes. These are not associated with myocardial damage and are reversible with the control of airway obstruction.[118] Finally, alveolar clearance measured by scintigraphy indicates an impaired alveolar epithelium integrity that persists in asymptomatic horses kept in a low dust environment and only return to normal when horses are on pasture.[114]

## AIRWAY INFLAMMATION

### Cytology of the lower airways

Characterization of disease-associated airway inflammation in human asthmatics relies primarily on non-invasive sputum induction. In horses, airway cells are sampled by saline instillation in the trachea, and by BAL. Increased neutrophil percentages in tracheal lavages are not specific for heaves, neither does it correlate with BALF cytology or histological changes.[119–122] Similarly, poor correlations between neutrophil percentages in sputum, BALF and bronchial biopsies are observed in asthma.[123] BAL are the preferred mean to assess the granulocyte populations in horses and are easily performed on standing sedated animals (Fig. 1b). In heaves, neutrophil percentages in BALF are usually 25% or more (Fig. 3b) (normal $\leq$ 5%). Changes in percentages rather than absolute counts are considered as fluid retrieval and dilution amplitude of the initial volume of saline instilled (up to

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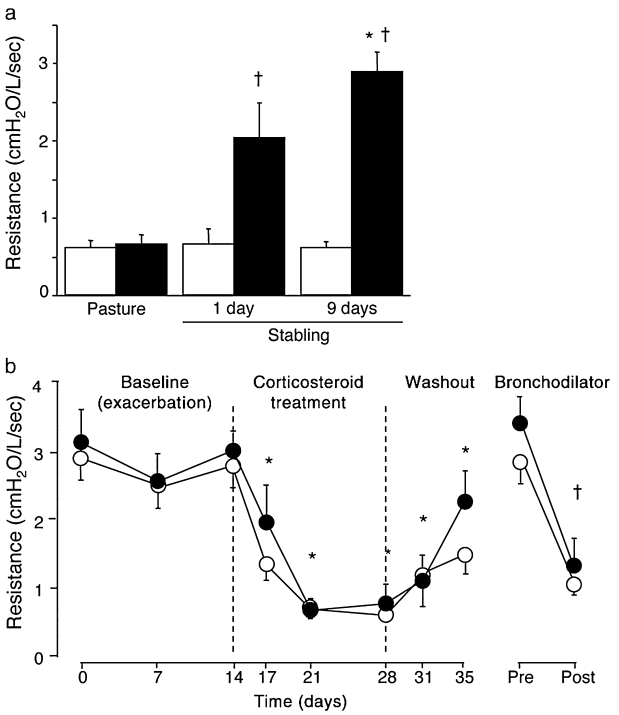


**Figure 2** (a) Pulmonary resistance of horses with heaves ($n = 6$) (black bars) and controls ($n = 6$) (white bars) before (Pasture) and after natural antigen exposure (Stabling and hay feeding) of 1 and 9 days duration. Mean ± SEM. *Different from pasture; †different between groups ($P < 0.05$). Reprinted from Cordeau *et al.*[55] with permission from Elsevier. (b) Changes in pulmonary resistance in horses with heaves before and following treatments with corticosteroids (dexamethasone ($n = 6$), open circles; isoflupredone acetate ($n = 6$), black circles) or a bronchodilator (atropine). Mean ± SEM. *Different from baseline; †different from pre bronchodilator treatment ($P < 0.05$). Reprinted from Picandet *et al.*[98] with permission from Wiley.

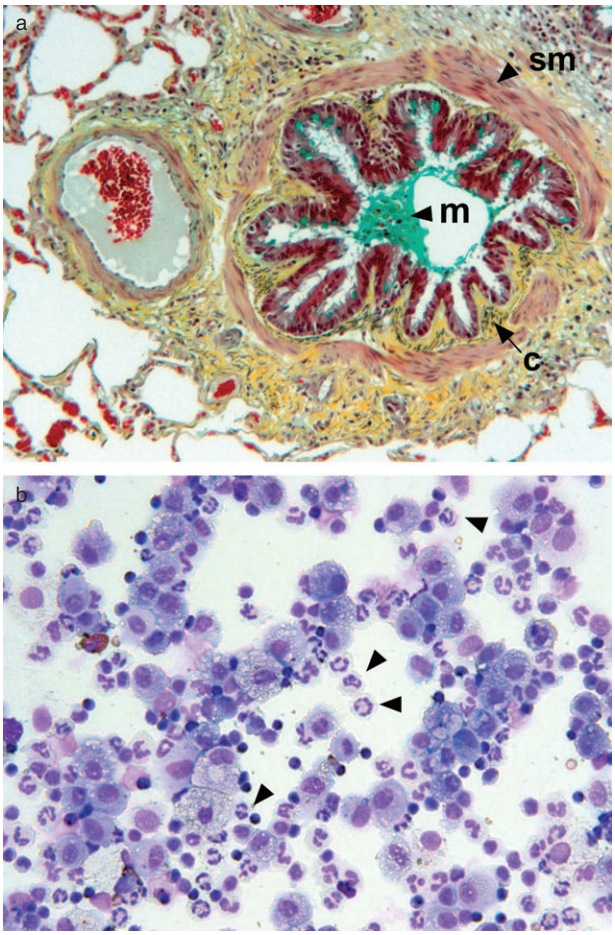


**Figure 3** (a) Representative histology of an inflamed bronchiole with mucus plug (m) and surrounding tissue remodelling (sm, smooth muscle; c, subepithelial collagen) in a peripheral lung biopsy obtained by thoracoscopy. Movat pentachrome staining, original magnification 10×. Courtesy of Dr Emilie Setlakwe. (b) An example of BALF cytology (modified wright-giemsa staining, original magnification 20×, some neutrophils are pointed with arrowheads) from a horse affected with heaves.

500 mL) vary with horses and disease severity.[124] Inconsistent increases in eosinophils and mast cells are also reported (reviewed by Robinson[11]).

## Neutrophils

Airway neutrophils are present in large numbers in the airways of horses with heaves and may contribute to the disease through the release of several inflammatory mediators. They are recruited 3–5 h following the beginning of exposure to stable dust, the process is reversible within 4 days after the cessation of a short exposure (5–7 h),[125–127] and up to 3 weeks or more under more natural conditions.[79] Resolution of airway inflammation coincides with neutrophil apoptosis and phagocytosis by alveolar macrophages.[125] Healthy horses may also develop airway neutrophilia when exposed to the same environment. However, it dissociates from altered lung function, is lesser than in horses with heaves, and usually resolves despite continuous exposure to stable dust.[55,128,129] In addition, neutrophils are more dense in heaves compared with controls, have delayed spontaneous apoptosis, and release elastase, oxygen metabolites, $LTB_4$ and probably MMP-9.[125,128,130,131] Equine neutrophils have been shown to express mRNA for cytokines relevant to pulmonary inflammation. Among them, IL-1β, tumour necrosis factor (TNF)-α, IL-8 and IL-17[58,59,63,64,132] are upregulated in heaves and hence could be derived from recruited neutrophils. NF-κB activity in BALF's neutrophils is increased[79,80] and their release of TNF-α and IL-1β has been proposed to contribute to sustained inflammation through autocrine and paracrine effects.[80] Increased pulmonary oxidative stress indices and hydrogen peroxide in exhaled breath condensate have been associated with BALF neutrophil percentages and tracheal neutrophil counts respectively.[133–135] Taken together, these


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results suggest that aberrantly high activation of airway neutrophils may participate to lung pathology in heaves. In addition, IL-4 induces a specific activation phenotype in equine neutrophils (increased expression of IL-4Rα, CD23/FcεRII, IL-8 and TNF-α), suggesting that they could contribute actively to allergic inflammation and to its regulation.[136] IL-4 may also participate in the recruitment of neutrophils by stimulating the release of adhesion molecules and chemotactic factors by equine endothelial cells.[137]

### Macrophages

The cellular density of BALF macrophages is increased after exposure to stable dust in horses with heaves compared with controls,[128] suggesting cellular activation. However, alveolar macrophages from both groups of horses have similar upregulated expression of selected pro-inflammatory cytokines (IL-1β and TNF-α) and neutrophil-directed chemokines (IL-8 and MIP-2) following stable dust challenge.[138] These results suggest that other mediators secreted by macrophages or different cell types are responsible for the overwhelming neutrophilic inflammation in heaves. Of interest however, percentages of neutrophils in BALF are correlated with IL-1β expression by total BALF cells[15,59] or alveolar macrophages,[138] suggesting that this cytokine contributes to the airway neutrophilia. IL-1β and TNF-α are produced by alveolar macrophages of normal horses stimulated *ex vivo* by *A. fumigatus*, suggesting that molecular patterns of mould antigens may favour cellular activation independently of clinical diagnosis.[139] Conversely, alveolar macrophages isolated from heaves-affected horses after inhalational challenges with endotoxin or HDS were found to have higher expression of TNF-α (HDS), IL-1β and IL-8 (endotoxin) than those of controls.[140] Early IL-17 and MIP-2 expression by pulmonary mononuclear cells in response to *ex vivo* stimulation with soluble hay dust also appears to discriminate between control and heaves-affected horses as reported in another study.[141] The latter suggests that early cytokine expression by resident macrophages might contribute to airway outcome.

### Epithelial cells

Airway epithelial cells are at the interface between the lung and the environment, and thus play a critical role in homeostasis. *In vitro*, IL-8, MIP-2 and IL-1β mRNA is upregulated in epithelial primary cell cultures from horses with heaves and controls in response to HDS or endotoxin.[142] However, only MIP-2 is overexpressed in cells from symptomatic heaves-affected horses compared with controls in response to HDS. There is increased IL-8 expression by epithelial cells in heaves compared with controls from day 14 following an organic dust exposure,[64] but not during acute exacerbation.[143] These observations suggest that epithelial cells may contribute to the chronic inflammatory response in heaves, but evidence of contribution at an earlier stage is currently lacking. Elevated expression of TLR4 was reported in bronchial brushings[143] in heaves but not in bronchial biopsies.[64] Increased TLR4 expression could lead to exaggerated innate immune response to hay dust endotoxin and contribute to the IL-8 expression associated with the disease.[143] Increased NF-κB activity is found in epithelial cells retrieved from bronchial brushings in heaves-affected horses and correlates with parameters of lung dysfunction and increased ICAM-1 expression by these cells.[80] Epithelial cells could therefore facilitate neutrophil transmigration towards the airway lumen through the upregulation of this adhesion molecule[144] and the expression of IL-17A.[64,141]

## SYSTEMIC INFLAMMATION

Peripheral blood alterations are associated with heaves exacerbation. A rise in haematocrit and fall in sedimentation rate were reported to occur almost 50 years ago.[3] There is a modest but significant increase in peripheral blood neutrophil 7–24 h following the initiation of stable dust exposure.[76,125,126] Blood neutrophils also have an increased active respiratory burst and delayed spontaneous apoptosis 24 h after challenge.[145–147] Increased CD18-dependant adherence is present, even during clinical remission, suggesting a persistent state of neutrophil activation.[145] Lymphocytes (phosphodiesterase (PDE) activity)[148] as well as platelets (decreased responsiveness to platelet-activating factor)[149] are also activated following natural antigenic challenge. In addition, blood concentrations of inflammatory mediators including endothelin,[150] thromboxane A2[151] and oxidation markers (reduced and total glutathione, GSH/TGSH)[152] are reported to rise with clinical signs.

Increased mRNA expression of IL-8 and TNF-α from both normal and heaves-affected horses' peripheral blood neutrophils has been reported following a 30-day stable dust exposure,[136] but this may not be an early event as no increase was observed at 5 h.[153] Peripheral blood viscoelastic properties and TNF-α protein are increased in symptomatic horses.[154,155] Systemic inflammation may persist during remission of the disease, as revealed by our recent observations that TNF-α remains elevated when horses are kept under low dust conditions (A. Lavoie-Lamoureux, K. Maghni and J.-P. Lavoie, 2009, unpubl. data). Using markers of the acute phase response, we found that haptoglobin and serum amyloid A protein (SAA) were increased following stable dust exposure, and that haptoglobin remains elevated after exposure cessation.[156] These markers are increased in the blood of asthmatic patients.[157–162] This finding is of clinical relevance, as systemic inflammation has been associated with greater decline in lung function in young healthy adults.[163] Heaves may therefore be a useful animal model to investigate the causes and consequences of systemic asthmatic inflammation.

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## MUCUS ACCUMULATION

In horses, mucus is primarily produced by cells lining the airways, while mucous glands play a lesser role.[164,165] Using tracheobronchial endoscopy and semi-quantitative scoring systems, tracheal mucoid secretions were shown to correlate with neutrophilic inflammation[166] and cough.[167] It increases during exacerbation of heaves and incompletely resolves with antigen withdrawal.[168] Mucus accumulates in both large and small airways, and plugs in alveoli and bronchioles may be observed (Fig. 4).[169,170] Although poorly described, severe and persistent mucus accumulation may lead to severe airway obstruction which may be refractory to therapy. Mucus glycoprotein composition is modified[171] and viscoelasticity increased[172] in BALF and tracheal secretions, respectively. In asthma, calcium-activated chloride channel-1 (CACL1) and mucin-5AC (MUC5AC) are signalling pathways possibly involved in mucus hypersecretion (reviewed by[173]). They are also overexpressed in heaves, although these findings are inconsistent.[169,174–177] TNF-α may participate to the increased mucus production, as it is increased in heaves and is a potent inducer of MUC5AC by equine epithelial cells.[178] Increased cell survival could be another pathway leading to mucus accumulation and cell metaplasia, as over 40% of mucous cells are Bcl-2-positive in heaves, whereas only 1% are positive in controls.[179]

## NEURONAL AND NEUROENDOCRINE CONTROL OF BRONCHOSPASM

Bronchospasm is a key feature of heaves.[87] As in other species, airway smooth muscle tone is controlled by the autonomic nervous system, both centrally and via local axonal reflex, through activation of receptors via circulating catecholamines and the non-adrenergic non-cholinergic (NANC) system. Bronchospasm in heaves is predominantly mediated via muscarinic (M) receptors[8] but there is no evidence of an exaggerated response of smooth muscle M2 or M3 receptors to acetylcholine stimulation,[180–183] which is similar to what is observed in asthma.[184,185] However, bronchospasm is possibly explained by an alteration of the prejunctional, inhibitory M2 receptors, which provide negative feedback to the release of Ach.[186] In addition, bronchorelaxation is also defective in heaves, due to decreased β-adrenergic receptors density and coupling efficiency to G-protein,[187] as well as altered NANC response.[183] Specifically, there is evidence of inhibitory NANC dysfunction during heaves exacerbation,[181,183] and an upregulation of the neuropeptide neurokinin A receptor at the airway smooth muscle level, increasing its bronchoconstrictive effects.[188]

The effects of acetylcholine are not limited to those of a neurotransmitter, as cholinergic anti-inflammatory pathways have recently been identified.[189] Our group observed that acetylcholine inhibits the expression of E-selectin and vascular endothelial growth factor by equine pulmonary artery endothelial cells stimulated by recombinant equine IL-4.[190] These findings suggest that the cholinergic anti-inflammatory pathway may modulate pulmonary inflammation and remodelling in heaves. Other mediators increased during heaves exacerbation, including serotonin, endothelin-1, histamine and leukotriene $D_4$, potentiate the response of equine smooth muscle cells to acetylcholine, or increase its release. However, while they may contribute to increased cholinergic airway tone, cysteinyl leukotrienes are not believed to be major contributors to bronchospasm as leukotriene antagonists are ineffective for the treatment of heaves.[127,161,191]

## MICROSCOPIC LESIONS AND AIRWAY REMODELLING

Inflammation and remodelling of the airway wall are characteristic features of heaves. A consequence of airway remodelling is incompletely reversible, or even irreversible airway obstruction, bronchial hyperresponsiveness and an accelerated decline in lung function. The microscopic lesions are mainly characterized by chronic bronchiolitis with predominantly lymphocytic and plasmacytic infiltration in the bronchiolar and peribronchiolar areas.[93,192–194] Mucus, neutrophils and cellular debris are commonly observed within the airway lumen.[143,192] Eosinophilic and mast cell infiltrates, as well as peribronchial fibrosis, are inconsistent findings. Lymphocyte aggregates around the vasculature of the small airways may also be seen.[193] Alveolar hyperinflation due to air trapping is common, while true emphysema is rarely present.[192,195] Changes are not uniform,[164,196] and may account for some variation in the reported microscopic descriptions. Some histological features of heaves, including increased smooth muscle mass and collagen deposition surrounding the airway as well as mucus accumulation in the airway lumen, are illustrated in Figure 3a.

### Epithelium

The main histological lesions in the large conducting airways are the loss of ciliated epithelial cells and occasional abnormal ciliated structures identified by electron microscopy.[197] In the smaller airways, mucous (or goblet) cell hyperplasia and metaplasia have been described[169,170] (and reviewed by Robinson[11]). Semi-quantitative analysis failed however to reveal significant differences in the number of mucous cells in the airways, while finding an association between stored mucosubstances and inflammation.[198] Clara cells that comprise up to 60% of the equine epithelium in distal bronchioles are almost completely depleted of their granules in heaves, and without concurrent observable mucus accumulation.[194] Non-uniformly distributed epithelial progenitor cell hyperplasia has been qualitatively described in studies using light and electron microscopy


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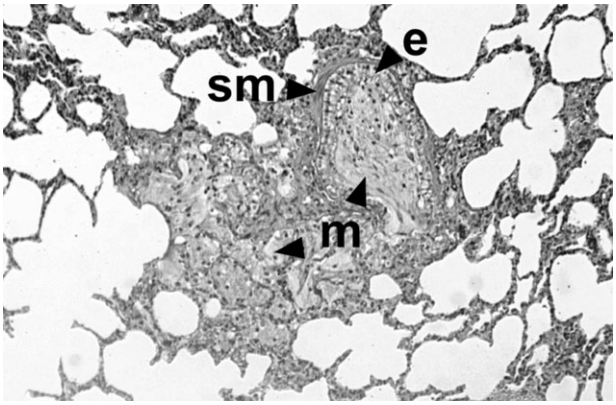


**Figure 4** Severe mucus accumulation within the lumen of a terminal bronchiole and surrounding alveoli of a horse with heaves refractory to therapy. m, mucus; e, epithelium; sm, smooth muscle. HE staining, original magnification 20×.

(reviewed by[11]). Finally, alveolar clearance remains abnormal during clinical remission in low-dust environment,[114] which suggests persistently impaired epithelial integrity.

### Airway smooth muscle

There are several reports in the early veterinary literature of smooth muscle hypertrophy and/or hyperplasia in horses with heaves.[117,170,195] Using morphometric techniques, our group has shown recently that an increase in airway smooth muscle mass is present in the airways of all calibres, but it is most marked in the distal airways (2 to 3 times the amount present in healthy age-matched controls).[129,199] Chronic smooth muscle remodelling appears to reach a plateau, which is possibly maintained in a dynamic equilibrium with an elevated turnover (increased proliferation and apoptosis) of airway myocytes.[129] As in human asthma, it is unknown whether chronic airway smooth muscle remodelling is reversible, although recent findings suggest that a modest but significant decrease in smooth muscle is seen with long-term treatment.[200]

### Collagen

Although not consistently found in the literature, pulmonary fibrosis (illustrated in Fig. 5) has been described in heaves using both light microscopy[12,13,93,193,195,196,201–203] or electron microscopy.[164] However, the collagen types involved in remodelling remain to be elucidated, as no differences in immunolabelled collagen types I and III was identified between healthy and asymptomatic heaves-affected horses in one study.[204]

### Tissue cellular infiltration

Although lymphocytic and plasmacytic infiltration predominates,[93,192–194] other types of cellular infiltrations have been reported, including neutrophil/eosinophil, neutrophil/lymphocyte, eosinophil/lymphocyte or even no cellular infiltration.[50] Of note, some control animals free of clinical signs present similar airway wall inflammation. Mast cells are increased in bronchial and bronchiolar airways in heaves-affected horses after challenge.[49,50] Infiltration with chymase$^+$ mast cells was associated with tissue fibrosis, neutrophil or lymphocyte but not eosinophil infiltration, independently of clinical diagnosis.[50]

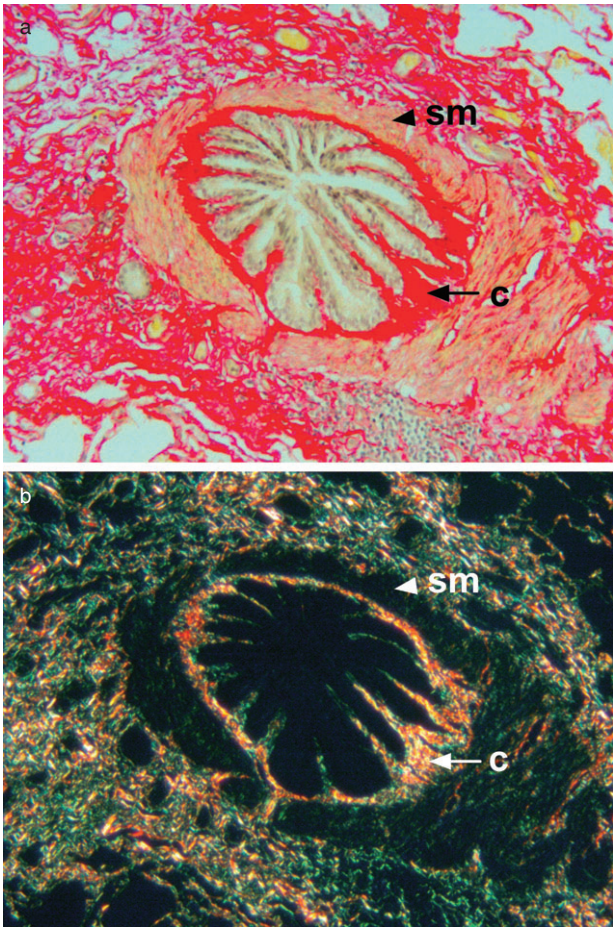


**Figure 5** (a) Peripheral equine airway stained with picrosirius-red. The subepithelial collagen area is stained red and is discernable from the surrounding smooth muscle layer. (b) Same airway under polarized light. Picrosirius-red staining, original magnification 10×. sm, smooth muscle; c, subepithelial collagen. Image courtesy of Dr Emily Setlakwe.

## TREATMENTS

There is no known cure for heaves, that is, susceptible horses recurrently develop airway obstruction and inflammation when exposed to offending environmental conditions. The corner stone of heaves management is thus organic dust avoidance; bronchodilators and corticosteroids are administered

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to provide rapid relief of airway obstruction, or when control of the environment is partial or absent.

## Organic dust avoidance: the most effective long-term approach

In heaves, as in allergen-induced asthma, removing the offending antigens is central to the control of the disease.[205,206] Grass pasture leads to alleviation of clinical signs, and normalization of conventional lung function tests and BALF neutrophilia.[129,207,208] Clinical signs subside in days to weeks, depending on age, and severity or duration of the disease.[209] In an indoor low-dust environment, subclinical inflammation and airway obstruction may persist however.[10,114,115] Horses with SPAOPD are conversely controlled by removing affected animals from pasture.[210]

## Medication with strong evidence of efficacy

Corticosteroids, whether administered systemically or by inhalation, are the most effective medication for the control of clinical signs and lung function in heaves[211] (and reviewed by Williamson and Davis[212]) (Fig. 2b). Partial and transient improvement may be observed within a few hours,[213] but the maximal control of airway obstruction usually requires a week or more.[59,191,214] If not combined with organic dust avoidance, only short residual effects are observed after drug administration is discontinued,[215] and control of pulmonary neutrophilia remains modest.[216–218] This is apparently not due to an inherent resistance of neutrophils to corticosteroids, as dexamethasone is very effective in inhibiting their genomic and non-genomic inflammatory responses *in vitro*.[219] In heaves, corticosteroids reduce IL-8 in BALF only if combined with antigen avoidance management,[59,208] and they do not affect NF-κB or AP1 in bronchial epithelial cells during stabling.[218] Thus, the release of neutrophilic chemotactic factors by airway structural and inflammatory cells possibly occur through NF-κB or AP1 signalling pathways, which appear to be resistant to corticosteroids in horses kept in an antigen-rich environment. These findings indicate that improvement in airway function with corticosteroids is likely the result of an anti-inflammatory effect on other cell types present in the equine airways, whether it is by preventing antigen-induced CD4+ T cells from increasing or by modulating the IFN-γ/IL-4 ratio towards a Th1 response,[59,216] or possibly by directly affecting smooth muscle contractility.[220]

Corticosteroids administered systemically to horses have been associated with some adverse effects (reviewed by Dauvillier *et al.*[221]) also observed with these drugs in human patients. On the immune system, they cause transient peripheral neutrophilia and lymphopenia,[222,223] alter the lymphocyte subpopulations and expression of activation markers,[224] and decrease the antibody response to vaccination.[225] On the contrary, when corticosteroids are administered by inhalation they have little systemic effects in horses with heaves, as shown by the absence of detectable clinical adverse effects or alteration of lymphocyte subpopulations and function, circulating neutrophil gene expression, and primary and anamnestic immune responses to vaccination after up to one year of inhaled fluticasone administration.[221]

Bronchodilators with demonstrated efficacy in horses with heaves include inhaled and systemic $\beta_2$-agonists and anticholinergic drugs. Short- and long-term inhaled $\beta_2$-agonist administration results in significant reduction in pulmonary resistance[226,227] (and reviewed by Williamson and Davis[212]). A recent study on *ex vivo* equine bronchial rings showed, however, different effects of the R and S-enantiomers of salbutamol,[228] echoing questions raised in people over the effects of different forms of $\beta_2$-agonists.[229,230] Clenbuterol, a systemic $\beta_2$-agonist, improves clinical signs in approximately 75% of affected horses,[231] but does not alter histamine-induced bronchoconstriction in healthy ponies.[232] Clinical response increases with higher dosage, as do adverse side-effects.[231] In addition to its bronchodilator effect, clenbuterol also increases mucociliary clearance[233] and possibly has some anti-inflammatory effects.[234,235] Recently, it was shown that corticosteroids can prevent clenbuterol-induced downregulation of $\beta_2$-adrenoreceptors on equine lymphocytes.[236] Inhaled anticholinergic drug ipratropium has been less studied but has dose-dependent effects.[9,237] Systemic anticholinergic agents are very effective at relieving airway obstruction (Fig. 2b), but are associated with systemic side-effects limiting their use as therapeutic agents.[238]

## Medication with mitigated or no efficiency in heaves

Methylxanthine derivatives are non-specific PDE inhibitors that have beneficial effects on lung function in horses with heaves but have low therapeutic index.[239–243] The potentiating effects of corticosteroids by low-dose theophylline reported in asthmatics[244] was not observed in horses with heaves after short-term administration.[241] In a recent study, a selective PDE4 inhibitor with potent *in vitro* anti-inflammatory effects on equine leukocytes failed to improve lung function and airway inflammation in heaves-affected horses.[217] Cilomilast, another selective PDE4 inhibitor, does not counteract methacholine-induced constriction in precision-cut lung slices.[245] Cysteinyl leukotrienes (LTC4, LTD4, LTE4) are potent bronchoconstrictors derived mainly from mast cells. They may also contribute to airway diseases by increasing airway vascular permeability and mucus production. Leukotriene receptor antagonists are alternative therapy for the treatment of mild persistent asthma.[205] LTD4 also causes bronchoconstriction both *in vitro* and *in vivo* in horses, and the main receptor for cysteinyl leukotrienes, CystLT1, is present on the equine bronchi.[191,246,247] However, a LTD4 receptor antagonist, a 5-lipoxygenase (LO) inhibitor and a 5-LO-activating protein antagonist


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**Table 1** Similarities and differences between heaves and rodent models of asthma

| Characteristics | Equine heaves | Rodent asthma models |
|---|---|---|
| Aetiology | Develops naturally<br>Influenced by gene-environment interactions<br>Natural antigens and routes of sensitization/challenge | Antigen-induced<br>Some of the antigens used (e.g. ovalbumin) and routes of sensitization/challenge (e.g. intra-peritoneal/trachea) are irrelevant to human asthma<br>Strongly bias by inbred genetics<br>Animals kept in pathogen-free environments |
| Pathophysiology | | |
| Non-specific AHR | Present | Present |
| Inflammation | Persistent<br>Neutrophilic with mixed Th1/Th2/Th17-cytokine profiles | Transient<br>Depends on the antigen (e.g. house dust mite/ovalbumin), adjuvant (e.g. *Bordetella pertussis*/alum) and mice/rat strain (e.g. BALB/c mice and Brown Norway rats are predisposed to Th2-driven atopic responses) |
| Tissue remodelling | Persistent, mostly distal airways | Transient, central and distal airways |
| Clinical signs | Airway obstruction, cough, nasal discharge, wheeze | Usually absent |
| Research setting | | |
| Genetic manipulation | Not feasible | Knock outs, transgenics, chimera |
| Reagents availability | Limited | Most current |
| Availability | Infrequent | High |
| Handling costs | High | Affordable |

were ineffective in heaves,[127,191,248,249] suggesting that cysteinyl leukotrienes are not an important mediator of acute bronchoconstriction in this disease.[191] Cromolyn sodium stabilizes mast cells and interferes with chloride channel function. It is used as alternative medication in mild asthma, and as preventive treatment before unavoidable exposure to known allergens.[205] In heaves, they also prevent clinical exacerbation, although their efficacy is variable.[250,251]

It has been suggested that beneficial effects of corticosteroids are partially mediated through down-regulation of the p38 mitogen-activated protein kinase (MAPK) pathway. Inhibition of p38 MAPK was partially effective in reducing clinical signs and airway inflammation when administered before clinical exacerbation in heaves.[252] However, they were ineffective when administered during clinical exacerbation, and severe side-effects were observed. Inhibitors of p38 MAPK pathways with a better toxicity profile might nevertheless be effective in the prevention or treatment of heaves, especially since this pathway is essential for equine neutrophils migration.[253]

## ADVANTAGES OF HEAVES AS A MODEL FOR ASTHMA

Equine heaves and feline asthma are perhaps the only naturally occurring asthma-like conditions affecting domestic animal species. They are both associated with inflammation of the airways, coughing and bronchoconstriction.[254] However, the study of feline asthma in a research setting usually requires experimental sensitization with *Ascaris suum* antigens,[255] as induction of natural exacerbation in affected cats is rarely achievable. In the future, the development of lung function testing, biopsy sampling and molecular tools better adapted to cats might make it a more useful model. Other models in rodents (Table 1), pigs, dogs, ruminants, primates (reviewed by Kirschvink and Reinhold[256] and Bice *et al*.[257]) also require experimental sensitization with an antigen administered systemically or by inhalation. They can be very useful in the study of specific aspects of asthma but rarely result in the development of persistent induced clinical signs and airway obstruction. The naturally occurring nature of heaves, the duration of the disease that can last decades and the long lifespan (30–35 years) of horses make this disease closer to human asthma than any other animal models. This is particularly important for the study of airway wall remodelling that is likely the result of complex immune response and bouts of inflammation and bronchoconstriction occurring over many years. Also pertinent to the study of airway remodelling, the equine and human lungs have similar well-developed bronchial circulation,[258–260] poorly developed lobulation and respiratory bronchioles, thick pleura, and the presence of terminal bronchioles and bronchial artery-pulmonary shunts.[260] Contrarily to small rodents, they have well-developed smooth muscle from the trachea to the alveolar ducts[261] and mast cells are present at all levels of the bronchial three.[262] An additional strength of this model is that episodes of airway obstruction can be predictably induced in susceptible horses by an exposure to stable dust and reversed by maintaining horses on pasture or by the administration of corticosteroids and bronchodilators.[59,263–265] Because of their calm and malleable demeanour, procedures required for the study of lung pathologies, such as

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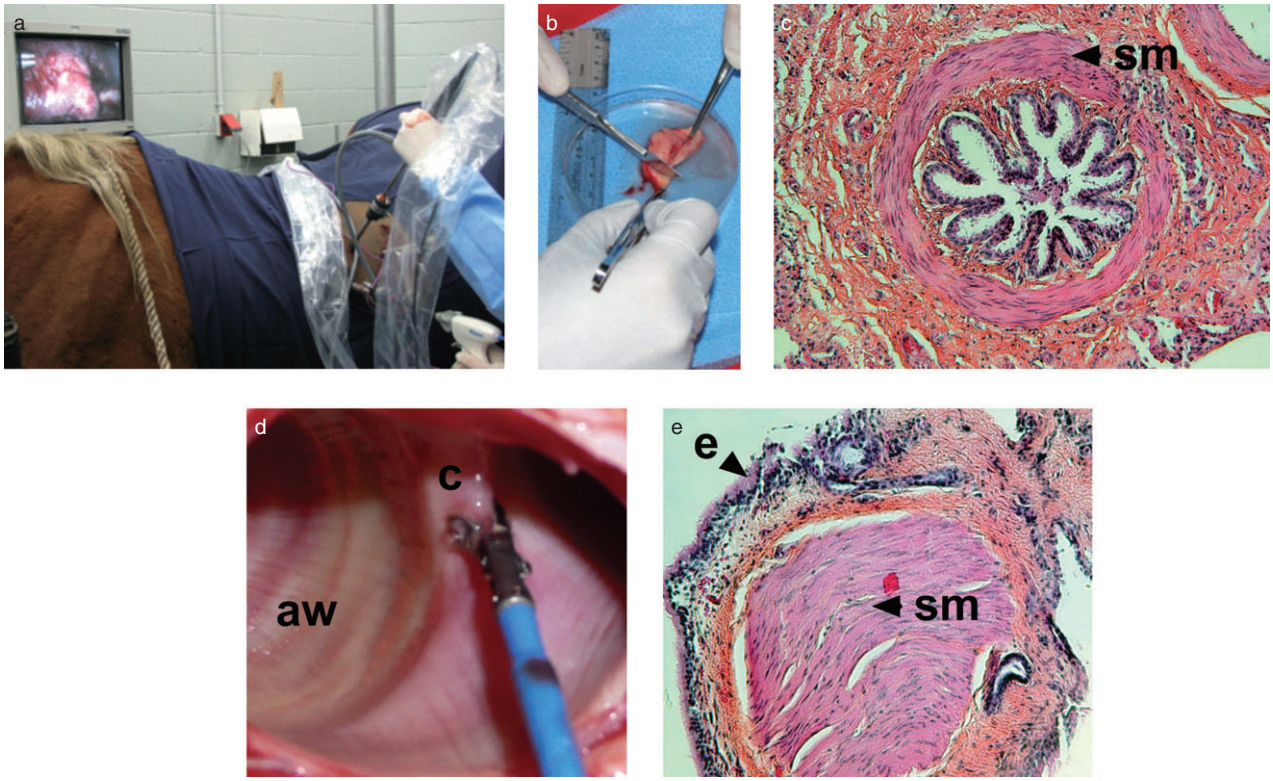


**Figure 6** (a) to (c) Peripheral lung biopsy obtained under thoracoscopy guidance in a heaves-affected horse. Thoracoscopy can be performed in standing, sedated animals, breathing spontaneously. Biopsies obtained contain numerous airways in cross-section like the one pictured here. sm, smooth muscle. Haematoxylin phloxine saffron staining, original magnification 10×. (d) and (e) Endobronchial biopsy obtained at the level of a secondary carina through a bronchoscope. The biopsies obtained sample a portion of the airway wall. c, carina; aw, airway; e, epithelium; sm, smooth muscle. Haematoxylin phloxine saffron staining, original magnification 4×.

pulmonary function tests, BAL and endobronchial biopsies (Fig. 6d,e) or brushing, and even large peripheral lung biopsies performed via thoracoscopy (Fig. 6a–c) can also be performed on multiple occasions in standing sedated animals.[266–268] Thus, the prospective study of equine cohorts allows the analysis of disease mechanisms and evolution of remodelling in the asymptomatic and symptomatic stages of disease, an approach difficult in humans. The recent development of owner-assessed respiratory signs questionnaires[17,211,269,270] also allows for conducting large-scale epidemiological studies and field evaluation of treatment efficacy.

The disease has initially been disregarded as an asthma model because neutrophils are the predominant cells in the airway lumen in heaves. Interestingly, it is now recognized that neutrophils infiltrate the airways of nocturnal asthmatics,[271] non-atopic and atopic asthmatics,[272,273] and are the predominant cells in the airway secretions of a significant proportion of asthmatics, ranging from mild to severe.[274–279] Similarly to asthma, there is a genetic susceptibility to heaves[18,20] and an allergic component is supported by the presence of a Th2-type pattern of cytokine expression in the airways[54,55,61] and an IL-4Rα gene polymorphism,[23] although, as in asthma,[280] a Th1 cytokine profile has also been reported.[58,59] Hence, heaves also serves as a model to study the contribution of neutrophils to the asthmatic phenotypes. Furthermore, horses present mild or moderate asthma-type conditions that are associated with different airway inflammatory phenotypes. Lastly, cross-bridging of the human and veterinary researches may benefit our understanding of both diseases. Table 2 summarizes the similarities and differences between asthma and heaves.

## LIMITATIONS OF HEAVES AS A MODEL FOR ASTHMA

Aside from the pathophysiologic differences intrinsic to any animal model, heaves comes with unique challenges. The natural nature of heaves and the heterogenic genetic background of the affected animals make it difficult to reach adequate statistical power when looking at small effects (in genetic studies for example). Also, the long lifespan of horses complicates longitudinal studies on ageing processes. However, these aspects represent advantages compared with rodents when it comes to model human diseases. Their size can be an asset for repeated biopsy sampling and ease of bronchoscopy but limits other procedures such as thoracic computed tomography. Interspecies differences in immune-related


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**Table 2** Similarities and differences between asthma and heaves

| Pathophysiological features | Asthma | Heaves |
|---|---|---|
| Reversible airway obstruction | ✓ | ✓ |
| Airway hyper-responsiveness | ✓ | ✓ |
| Acute phase | ✓ | |
| Late phase | ✓ | ✓ |
| Neutrophilic inflammation | ✓† | ✓ |
| IgE-specific response | ✓† | ? |
| Inflammation profile | | |
| Th2 and/or Th1 | ✓ | ✓ |
| Th17 | ✓‡ | ✓ |
| ↑ Endotoxin sensitivity | ✓ | ✓ |
| Inflammation site | | |
| Peripheral airways | ✓ | ✓ |
| Central airways | ✓ | ? |
| Tissue remodelling | | |
| ↑ Smooth muscle mass | ✓ | ✓ |
| Submucosal fibrosis | ✓ | ✓ |
| Basal membrane fibrosis | ✓ | |
| Remodelling sites | | |
| Peripheral airways | ✓ | ✓ |
| Central airways | ✓ | ✓ |
| Treatments | | |
| Corticosteroids | ✓ | ✓ |
| $\beta_2$-agonists | ✓ | ✓ |
| Anti-leukotrienes | ✓ | |
| Theophylline | ✓ | ✓ |

? No clear conclusion.
† Some phenotypes.
‡ Associated with severe phenotypes.

mediator or receptor expression as well as dissimilarities in drug and nutrient metabolism may restrain extrapolation to humans. Also, some molecular tools, in particular species-specific antibodies are still lacking despite constant improvement. Lastly, cost, facilities and equipment required for the study of horses has limited in the past the study of heaves as an asthma model. New means of increasing access to resources and collaboration between centres have been developed (see below) and will reduce some of those limitations.

## PERSPECTIVES

Heaves has unique features of interest for asthma research. For example, there are ongoing studies of airway remodelling reversibility that cannot be conducted in rodents or human for technical or ethical reasons.[200] Post-mortem and biopsy tissue availability facilitates *ex vivo* techniques such as precision cut slices,[245] primary smooth muscle culture, myosin purification and respiratory epithelial cell cultured on air-liquid interface.[281] Areas of particular interest include the genetic determinants of heaves,[282] the characterization of T-cell differentiation[283] and regulation,[284] the description of dendritic cell properties[285] and modulation,[286] the contribution of epithelium to inflammation,[142,143,287] as well as the role of surfactant[288] and Clara cells.[194] Our group and others are also interested in the mechanisms of neutrophil recruitment,[289] their contribution to Th2-derived inflammation,[136] and their responsiveness to corticosteroids.[290] These fields target a better understanding of asthma-like disease aetiology, progression and treatment improvement. Investigating the link between IAD and heaves, as well as how inflammation of the airways in younger horses evolves to heaves' severe neutrophilic inflammation and airflow limitation may help shed light on the biology of human asthma.

Veterinary schools worldwide are studying heaves and IAD as human disease models but also because they are of considerable importance for animal welfare and the equine industry. A multicentre equine respiratory tissue bank (http://www.btre.ca, October 2011) is being developed to increase availability of equine lung tissues to other researchers in the field of asthma that do not have the resources and expertise required to study this animal model.

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# Annexe 2

**Evaluation of a Thoracoscopic Technique Using Ligating Loops to Obtain Large Lung Biopsies in Standing Healthy and Heaves-Affected Horses**

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Sommaire

Cet article décrit la procédure chirurgicale utilisée pour le prélèvement des biopsies lors l'étude I. La nouveauté de cette procédure effectuée par thoracoscopie vient de l'utilisation de lassos pré formés qui sont complètement résorbables et peu coûteux, ce qui les distingue des agrafes chirurgicales utilisées jusqu'ici. Une autre nouveauté est d'avoir effectué les biopsies sur des chevaux atteints du souffle symptomatique. Les principales complications observées sont également décrites et incluent un glissement du lasso, de l'hypoxémie, des pneumothorax intra ou postopératoires et, rarement, des saignements pulmonaires. Toutes les complications ont été traitées adéquatement et n'ont pas affecté la survie des animaux.

**Contribution**
J'ai contribué à l'organisation du transport et de la préparation des chevaux (80%), à la coordination des différentes équipes (chirurgiens, anesthésistes, techniciens, équipe de récolte des tissus) (75%), à la sédation et au monitoring des chevaux (50%), à l'aspiration des pneumothorax controlatéraux pendant les procédures (80%), au suivi postopératoire (90%) et à la rédaction de l'article (10%).

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# Evaluation of a Thoracoscopic Technique Using Ligating Loops to Obtain Large Lung Biopsies in Standing Healthy and Heaves-Affected Horses

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**Objective**—To evaluate use of pre-tied ligating loop to perform thoracoscopic, large lung biopsy in normal and heaves-affected horses.
**Study Design**—Prospective clinical study.
**Animals**—Normal (n = 5) and heaves-affected (n = 6) horses.
**Methods**—Lung biopsies, 1 from each hemithorax, were collected thoracoscopically using a pre-tied ligating loop. Horses were either normal (C) or heaves-affected with the latter being in remission (Ha) for the initial biopsy and in exacerbation (Hs) for the 2nd biopsy. Clinical variables, $PaO_2$, and $PaCO_2$ were used to determine the effect of surgical biopsy. Postoperative pneumothorax was monitored by serial thoracic radiographic examinations.
**Results**—Thoracoscopic lung biopsy (n = 29, 22 procedures) was well tolerated by all horses. Complication rate was 31%, including 8 ligature slippage and 1 pulmonary hemorrhage. Intranasal oxygen was administered intraoperatively to 6 horses (2 C, 1 Ha, 3 Hs) with severe hypoxemia or labored breathing. There was a significant decrease in $PaO_2$ during surgery in horses not supplemented with oxygen. Postoperative pneumothorax (21/22 procedures) detected radiographically resolved within 3 weeks.
**Conclusion**—Thoracoscopic lung biopsy using pre-tied ligating loops was minimally invasive, relatively inexpensive, and fairly efficient. Heaves-affected horses tolerated the surgery well, even when in exacerbation; however, the technique was associated with non life-threatening complications in 31% of the biopsies, most of which required correction with additional ligating loops or more sophisticated instrumentation.
**Clinical Relevance**—Using laparoscopic pre-tied ligating loop for thoracoscopically-assisted lung biopsy can be considered in horses with normal and impaired lung function but alternative instrumentation and access to intranasal oxygen must be available to the surgeon in case of complications.
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## INTRODUCTION

EQUINE LUNG biopsy was initially reported using a trephine percutaneously inserted in anesthetized horses.[1,2] Subsequently, a Tru-Cut biopsy needle and an automated biopsy needle have been used in sedated horses.[3–9] In a survey on percutaneous lung biopsy, 77% of large animal Diplomates of the American College of

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Veterinary Internal Medicine reported complications including epistaxis, pulmonary and great vessel hemorrhage, pale mucous membranes, tachycardia, tachypnea, respiratory distress, collapse, pneumothorax, and death.[7] Despite these complications, biopsies were histologically representative of tissue and horse disease, including heaves also known as recurrent airway obstruction (RAO).[4,5,8]

Thoracoscopic anatomy in standing horses, for diagnostic and therapeutic purposes, has been described.[10–13] Thoracoscopic lung biopsy technique using biopsy forceps has been used in several species[14,15] including horses[13] with harvested samples being representative of the patient's condition. Nevertheless, although not reported in horses,[13] this inexpensive and safe technique may require specific equipment like electrosurgery to control hemorrhage or air leakage from the biopsy site.[15] Thoracoscopic lung biopsy technique using endoscopic staples was reported in heaves-affected horses in clinical remission.[16] The caudal tip of the lung was sampled and specimens from the lung periphery were histologically useful for diagnosis of chronic pulmonary disease. Reported complications with this technique included minor discomfort during manipulation of the thoracoscope, postoperative hemothorax, and pneumothorax; however, endoscopic staples are expensive, which limits their use. A more affordable material could be useful for repeated clinical or experimental lung biopsies in horses.

Surgical indications for using an endoscopic pre-tied ligating loop include standing or recumbent laparoscopic cryptorchidectomy[17–19] and ovariectomy.[20–23] Hemostasis is achieved by compression, when tightening the loop around the mesorchium or mesovarium. Pre-tied ligating loops have also been used for laparoscopic removal of granulosa cell tumors,[24,25] and although vessel size increases in tumoral tissues, complication rates seemingly do not differ from those observed during routine ovariectomy.[24,25] In addition, this affordable device ($\sim$ 5 times cheaper than laparoscopic staples) has been used without major complications for thoracoscopic lung biopsy in humans[26] and clinically normal dogs.[14,27]

We hypothesized that an endoscopic pre-tied ligating loop used for thoracoscopic lung biopsy would be a safe and efficient technique in normal horses and horses with compromised respiratory function. Our objectives were to evaluate use of a ligating loop to collect a large lung biopsy, especially the loop's ability to create a pedicle when tightening around a flat lung region, to determine tolerance of a thoracoscopic surgery in heaves-affected horses during exacerbation, and to evaluate biopsy tissue quality. This study was conducted as part of a larger study on heaves in horses.

## MATERIALS AND METHODS

### *Case Selection*

Adult horses (n = 11; 9 mares, 2 geldings; mean [± SD] weight, 486 ± 50.5 kg) were used; 6 horses had heaves, 5 were clinically normal controls. Two thoracoscopic lung biopsies, 1 in each hemithorax were performed. Before the 1st surgery, all horses were kept on pasture and control and heaves-affected horses were without clinical signs of respiratory disease. One month before the 2nd surgery all horses were stabled in a dusty environment, fed hay, and bedded on straw. Control horses remained free of signs of respiratory disease but heaves-affected horses developed signs of airway obstruction characterized by nasal flaring and increased abdominal effort. These respiratory signs were translated into clinical scores.[28] For nasal flaring, scoring was: 1 = no flaring; 2 = slight, occasional flaring of nostrils; 3 = moderate nostril flaring; and 4 = severe, continuous flaring during each respiration. For abdominal effort, scoring was: 1 = no abdominal component to breathing; 2 = slight abdominal movement; 3 = moderate abdominal movement; and 4 = severe, marked abdominal movement. Both scores were combined to yield a clinical score where a score of 2 = normal; 3 or 4 = mild signs; 5 or 6 = moderate signs; and 7 or 8 = severe signs. Clinical scores for heaves-affected horses at time of 2nd biopsy were $\geq$6.

### *Surgical Procedure*

Horses were restrained in stocks and sedated with intravenous (IV) detomidine HCl infusion (loading dose [LD] 6 μg/kg; continuous rate infusion [CRI] 0.8 μg/kg/min for 15 minutes and a half-decrease every 15 minutes thereafter),[16,29] with additional analgesia provided by pre-operative administration of butorphanol (0.02 mg/kg IV).

One horse from each group (control, heaves-affected) was chosen on procedure days; biopsies were collected from the same hemithorax (randomly selected for the initial procedure) of each horse. The opposite hemithorax of each horse was used for the 2nd procedure The thorax was clipped and aseptically prepared for surgery. After 2% lidocaine infiltration (15–20 mL), the skin was incised at the 13th intercostal space (ICS), 5 cm below the *erector spinae* ventral limit as previously described.[16] A teat cannula was introduced into the hemithorax through the incision until air aspiration was heard, signaling induction of pneumothorax. Then, a 100 mm length, 10 mm diameter reusable cannula (RIWO-ART™ Trocar Sleeve 10 mm, 100 mm length, Richard Wolf Medical Instruments Corporation, Milton, ON, Canada) with a trocar was introduced into the incision and through the thoracic wall. Care was taken to move away from the caudal aspect of the cranial rib to avoid the neurovascular bundle. A rigid 33 cm length, 0° laparoscope (Laparoscope MILLENIUM™, 0°, 10 mm, V. Mueller® Product, Cardinal Health, Toronto, ON, Canada) was inserted into the cannula and the hemithorax explored. Afterwards, 2 instrument portals were created in a similar manner to the thoracoscope portal with a 10 mm diameter cannula: 1 at the 12th ICS for the ligating loop

(SURGITIE™, Polysorb Size 0 USP, 21 inches, VIOLET, Autosuture Company, Tyco Healthcare, Pointe Claire, QC, Canada) and another at the 15th ICS for atraumatic laparoscopic forceps (ENDO SHEARS™, 5 mm, Autosuture Company), 5 cm ventral to the thoracoscopic portal (Fig 1).

Two surgeons (F.R., F.D.) collected the lung biopsies. The ligating loop was introduced through the 12th ICS portal, then atraumatic laparoscopic forceps through the 15th ICS portal. The forceps were passed through the ligating loop and the caudodorsal tip of the lung was grasped, stretched slightly to allow the loop to engage lung tissue, then the loop was progressively tightened (Fig 2). The lung was cut at least 5 mm distal to the ligature using laparoscopic scissors (ENDO SCIZ™, 10 mm, Autosuture Company) introduced through the 12th ICS cannula, then the sample was exteriorized by removing the forceps and cannula from the 15th ICS incision because the sample would not pass through the cannula. The precise site of biopsy was recorded and the surgical site was carefully evaluated for bleeding or ligature slippage once the biopsy was released (Fig 3).

The size of the biopsy was subjectively estimated. As multiple analyses were required for paired studies, at least 3 $cm^2$ of lung tissue were required. For histologic analysis only, 1 $cm^2$ was considered sufficient. Thus, if the biopsy was considered too small ($<3\,cm^2$) for the full panel of analyses another biopsy was taken and was counted as a separate biopsy procedure for statistical analysis.

During surgery, the presence of a contralateral pneumothorax was assessed by observation of the contralateral hemithorax through the dorsal mediastinum. Absence of contralateral pneumothorax was characterized by a contralateral lung tightly pressed against the mediastinum. Presence of contralateral pneumothorax was characterized by air visible between the mediastinum and the contralateral lung or by an inability to observe the collapsed contralateral lung.

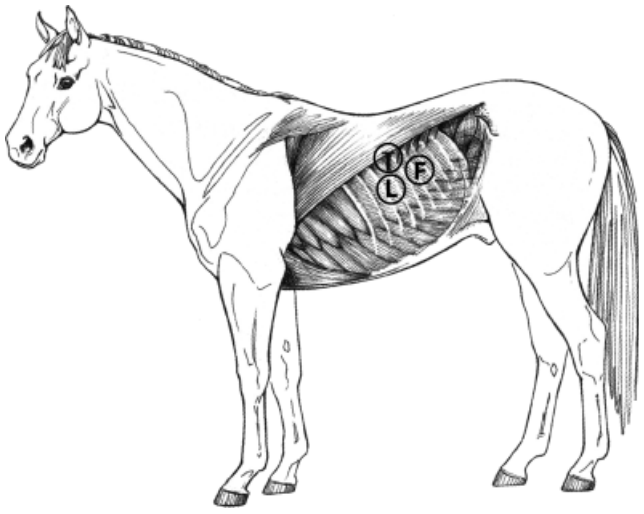


**Fig 1. Schematic view of the left thorax of a horse. Thoracoscope portal (T) at the 13th intercostal space (ICS), forceps portal at the 15th ICS (F), ligature portal at the 12th ICS (L).**

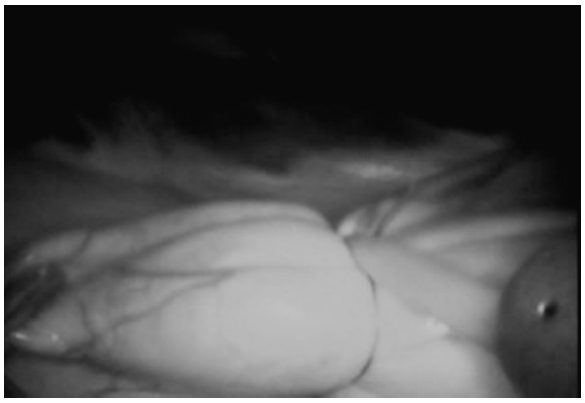

**Fig 2. Tightening of the ligating loop around the caudodorsal border of the right lung, which has been grasped with atraumatic forceps.**

At the end of surgery, skin instrumental portals were closed in a single layer. The lung was partially reinflated by aspiration of air present in the thorax under thoracoscopic observation with a surgical suction unit connected to the thoracoscopic cannula, which was removed as the lung inflated, as previously described.[16] A pre-placed purse string suture was immediately tightened as the cannula was removed. When contralateral pneumothorax was present, a teat cannula was introduced into the dorsal aspect of the 13th ICS on that side to re-expand the lung; air aspiration was considered sufficient when cannula movement in that hemithorax was limited by lung pressure. Sterile gauzes and an adhesive drape were used to keep the wounds protected for 24 hours. Surgical time, defined from initial incision to final suture closure, and complications, were recorded.

Procaine penicillin (22,000 U/kg IM) and phenylbutazone (2.2 mg/kg IV) were administered to each horse at the end of the surgery and continued twice daily for 2 days. Research protocol constraints prevented their administration before biopsy.

### *Intraoperative Evaluation*

During surgery, heart and respiratory rates were evaluated every 5 minutes. Arterial blood gas analysis ($PaO_2$ and $PaCO_2$) were performed at least 3 times during surgery: immediately after sedation, 15 minutes after induction of pneumothorax, and at the end of the surgery. Arterial blood sample was collected from the facial artery with a preheparinized 1 mL tuberculin syringe and immediately processed in a blood gas analyzer (Stat 7 Profile Analyzer, Nova Biomedical, Waltham, MA). Additional analyses were performed at the anesthesiologists (G.B., M.L.) discretion.

### *Postoperative Care*

Horses were stabled and monitored by continuous visual evaluation for the first 24 hours, after which they were returned to pasture. Clinical examination was performed at

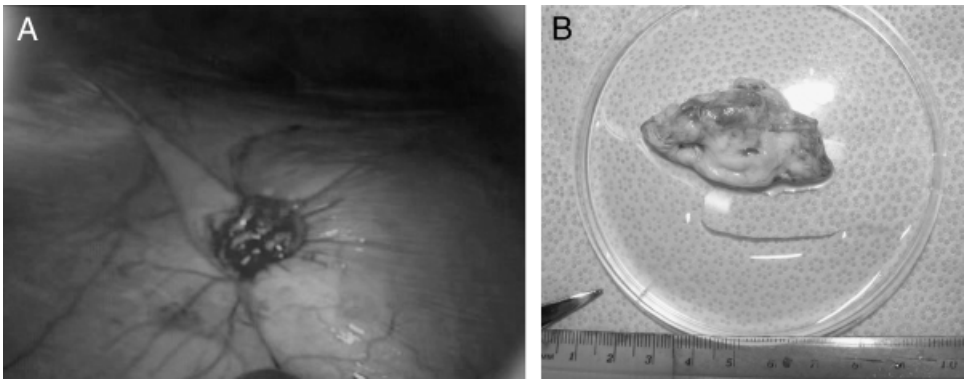


**Fig 3. Biopsy site appearance at the end of surgery (A) and the corresponding biopsy sample (B).**

2 and 12 hours postoperatively, twice daily for 2 days, and once daily for 5 additional days.

### *Radiographic Examination*

A complete left lateral thoracic radiographic examination (caudodorsal, caudoventral, craniodorsal, cranioventral projections)[30] in full expansion was performed preoperatively, and again at 2, 24, and 72 hours postoperatively.[27] Pneumothorax (free air in one or both pleural spaces dorsally), pneumomediastinum (air outlining the trachea, esophagus, or aorta) or pleural effusion (fluid in the pleural space ventrally)[31] were assessed by an evaluator unaware of the horse's condition, time pre- or postoperatively, or biopsy location. Time of maximal pneumothorax was determined by measuring (cm) the quantity of air present in the caudodorsal aspect of the pleural spaces at its maximal dorsoventral width. The side of maximal pneumothorax could be determined in most bilateral cases, because the left pulmonary margin having a more distinct margin than the right margin.[31] If pneumothorax was not resorbed after 72 hours, a caudodorsal projection only was repeated at a minimum of 1 week intervals until resolution of the pneumothorax. If an exacerbation of pneumothorax was noticed between 24 and 72 hours examinations, or if pneumothorax was associated with clinical signs of altered respiration (increased respiratory rate, respiratory distress) air aspiration was performed using a teat cannula, as described earlier.

### *Statistical Analysis*

For statistical analysis, horses were assigned to 3 different groups: control horses (C); heaves-affected horses in remission (Ha); and heaves-affected horses in exacerbation (Hs).

An exact $\chi^2$ test was used to determine the relationship between groups, biopsy site and complication rate, and between the presence of intraoperative pneumothorax and group. A repeated-measures linear model, with time and surgery number as within-subject factors and heaves status as a between-subject factor, was also used to compare clinical variables and arterial blood gas analysis between the 3 different times for the different groups. A repeated-measures linear model, with surgery number as a within-subject factor and heaves status as a between-subject factor, was used to examine differences in surgery time between the 3 groups. The level of significance was set at $P < .05$ for all analyses.

## RESULTS

### *Surgical Procedure*

Mean ($\pm$ SD) surgical time was $42.0 \pm 17.4$ minutes (range, 15–95 minutes) with no significant difference detected between the 3 groups.

Twenty-nine biopsies were collected; 7 biopsies were too small ($< 3\,cm^2$) for the paired studies; however, they were large enough ($\geq 1\,cm^2$) for histologic analysis. Biopsy samples contained multiple vessels and airways ($\sim$5–20 airways in cross section, with areas ranging from $<$0.1–2 mm diameter.) with well preserved pulmonary architecture and multiple inflated alveoli.

Complications occurred with 12 biopsies, specifically 8 ligature slippages (2 C, 2 Ha, 4 Hs; Table 1), 1 pulmonary vessel hemorrhage (1 Ha), 2 intercostal hemorrhage (1 C, 1 Hs) during portal cannula insertion, and 1 horse had a sudden movement during the procedure because of deep sedation (1 C). Intercostal hemorrhage and the sudden movement were considered to be associated with the surgical procedure but not specifically the biopsy technique. Thus, the complication rate related to use of pre-tied ligating loops for pulmonary biopsy was 31% (9/29).

When slippage of a ligating loop occurred, another pre-tied ligature (2 C, 4 Hs) or laparoscopic staples (2 Ha, 1 Hs; ENDO GIA™ ROTICULATOR™ 45–4.8 SULU, Autosuture Company)[16] were used to close the lung defect. One Hs horse required both pre-tied ligature and laparoscopic staples. Pulmonary hemorrhage was corrected by placement of a 2nd pre-tied ligature around the bleeding site (1 Ha). When intercostal hemorrhage was identified, the cannula was removed and the incision was

Table 1. Summary of Pneumothorax and Intraoperative Complications in 11 Horses (22 Surgical Biopsies)

| | | | | Maximum Pneumothorax | | | Complications | |
|---|---|---|---|---|---|---|---|---|
| Horse | Status | Site | Pneumothorax | Time (hours) | Side | Time of Resolution (days) | Type | Correction |
| 1 | C* | L | Bilateral | 2 | L | ND | | |
| | C†* | R | Bilateral | 2 | L | 7 | | |
| 2 | Ha† | L | Unilateral | ND | ND | 14 | | |
| | Hs‡ | R | Bilateral | 24 | L | 14 | Slippage | Loop + staples |
| 3 | C | R | Bilateral | 24 | R | 7 | | |
| | C† | L | Unilateral | 2 hours | ND | 1 | | |
| 4 | Ha‡ | R | Bilateral | 24 | R | 21 | Pulmonary bleeding | Loop |
| | Hs* | L | Unilateral | 72 | L | Aspiration | Intercostal bleeding | Staples§ |
| 5 | C* | L | Bilateral | 24 | ND | 21 | | |
| | C | R | Bilateral | 24 | L | 14 | Slippage | Loop |
| 6 | Ha | L | Bilateral | 24 | L | 21 | Slippage | Staples |
| | Hs†‡ | R | Bilateral | 24 | L | 21 | | |
| 7 | C‡* | R | Bilateral | 2 | R | 1 | Deep sedation | |
| | C† | L | Unilateral | 24 | L | 7 | Slippage | Loop |
| 8 | Ha | R | Bilateral | 24 | L | 21 | | |
| | Hs† | L | Unilateral | 24 | L | 21 | Slippage | Loop |
| 9 | Ha* | R | Unilateral | 2 | ND | 3 | | |
| | Hs* | L | Unilateral | 2 | ND | 3 | Slippage | Loop |
| 10 | C‡* | L | Bilateral | 2 | R | 21 | Intercostal bleeding | |
| | C | R | Bilateral | 2 | L | 14 | | |
| 11 | Ha | L | No pneumothorax | | | | Slippage | Staples |
| | Hs†‡ | R | Bilateral | 72 | L | Aspiration | Slippage | Loop |

*Bilateral pneumothorax detected intraoperatively.
†Biopsy too small for paired studies.
‡Intranasal $O_2$ administration intraoperatively.
§Staples were used to reduce surgical time.
C, Control horses; Ha, heaves-affected horses in remission; Hs, heaves-affected horses in exacerbation. ND, not determined. Staples.

enlarged dorsally. Bleeding from the intercostal site was stopped by application of 2 ligatures dorsally and 1 ventrally around the caudal intercostal neurovascular bundle using an absorbable suture. In 1 Hs horse with severe intercostal hemorrhage, staples were used to collect the lung biopsy to decrease surgical time. Detomidine infusion was stopped in the horse considered to be excessively sedated. No effect of group, horse status (remission, exacerbations) or biopsy site was detected on complication rate. During surgery, bilateral pneumothorax was identified in 8 procedures (5 C, 1 Ha, 2 Hs; Table 1).

### *Intraoperative Monitoring*

For control horses, respiratory rate increased significantly during ($P = .03$) and at the end of the surgery ($P = .005$) compared with baseline values. For heaves-affected horses, no significant effect of time or horse status (remission, exacerbation) was observed on respiratory rate.

Intranasal $O_2$ (15 L/min) was administered to 6 horses (2 C, 1 Ha, 3 Hs; Table 1) because $PaO_2$ was $< 60$ mmHg or respiratory distress occurred. Oxygen was administered intraoperatively to 5 horses, and preoperatively to 1 Hs horse with severe respiratory distress. During surgery in these 6 horses, mean $PaO_2$ and respiratory rate postadministration of $O_2$ were 97.2 ($\pm$ 49.1) mmHg and 21 ($\pm$ 7.2) breaths/min respectively. For statistical analysis, these horses were excluded because of the modification of $PaO_2$ induced by intranasal $O_2$ administration. For control and heaves-affected horses that were not administered supplemental $O_2$, there was a significant decrease in $PaO_2$ (C: $P = .0002$; Hs and Ha: $P = .02$) during surgery. For heaves-affected horses, a significant decrease in $PaO_2$ ($P = .03$) occurred in group Hs compared with group Ha at both time points of the procedure. No temporal ($P = .29$) or group ($P = .30$) effect on $PaCO_2$ was detected (Fig 4).

### *Radiographic Findings*

Postoperatively, bilateral pneumothorax was identified after 16 procedures (9 C, 3 Ha, 4 Hs; Fig 5), unilateral pneumothorax after 5 procedures, and no pneumothorax after 1 procedure. Pneumomediastinum was identified after 8 procedures and slight pleural effusion after 1 procedure. Pneumothorax resolved spontaneously within 3 weeks for 20 horses. The peak in pneumothorax severity

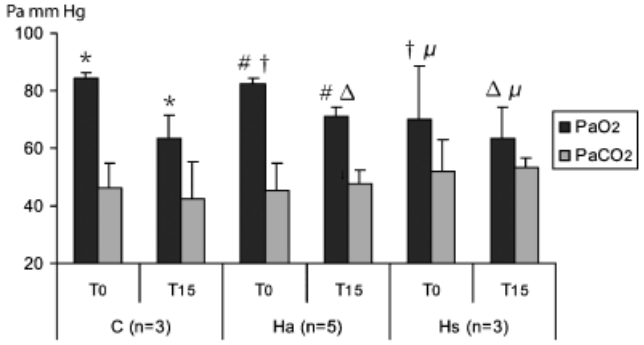


**Fig 4. Mean ($\pm$SD) $PaO_2$ and $PaCO_2$ mmHg at start of surgery (T0) and 15 minutes after induction of pneumothorax (T15) for normal horses (C), heaves-affected horses in remission (Ha) and heaves-affected horses in exacerbation (Hs). Horses administered intranasal $O_2$ supplementation were excluded from analysis. *, #, †, Δ, μ: significant difference ($P < .05$) compared with baseline values. (A–F): significant difference ($P < .05$) between bars.**

was noted at 2 hours for 8 horses (6 C, 1 Ha, 1 Hs), at 24 hours for 10 horses (4 C, 3 Ha, 3 Hs), and at 72 hours for 2 horses (2 Hs). Air was aspirated again in these 2 horses, because severity had increased between 24 and 72 hours. Radiographic examination was not possible for 1 horse at 2 hours because of technical problems and this horse was excluded from analysis of radiographic results.

The side with maximum pneumothorax was the same as surgery for 8 procedures, and the opposite for 8 procedures. The side with maximum pneumothorax could not be determined in 5 procedures (Table 1).

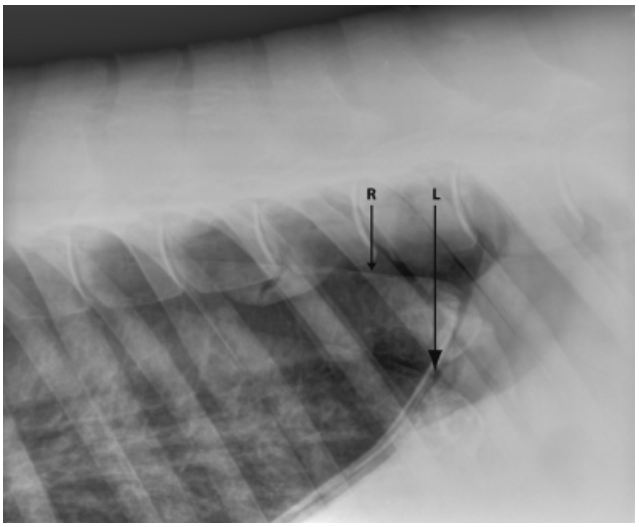


**Fig 5. Postoperative bilateral pneumothorax on a left lateral projection. Pneumothorax was more severe on the left side. Spondylosis deformans ventral to the T13–T14 intervertebral disc space is also present. R, dorsal edge of the right pleura; L, dorsal edge of the left pleura.**

*Postoperative Care*

One horse developed a superficial wound infection at 5 days; however the wound healed uneventfully by second intention after removal of skin sutures and wound lavage. Mild focal subcutaneous emphysema occurred at the surgical site after 20 procedures, and resolved spontaneously in a few days. One horse had decreased appetite with increased heart and respiratory rates for 3 days postoperatively; phenylbutazone (2.2 mg/kg orally every 12 hours) was administered for 2 additional days, after which its condition improved.

## DISCUSSION

Use of biopsy forceps yields representative lung samples in dogs and cats,[14] pigs,[15] and horses[13]; however, electrosurgery may be needed to reduce risk of pulmonary bleeding, pneumothorax, and prolonged air leakage.[15] Because of small sample size collected by biopsy forceps, multiple samples may be needed to confirm a diagnosis and potential artifacts created during collection could limit the quantity of interpretable tissue. Likewise, use of electrosurgery increases tissue thermal damage and may cause artifacts in the biopsy sample. Considering these concerns, we favored a hemostatic technique that would avoid thermal damage and so used a ligating loop technique as reported in humans, pigs, and dogs.[14,15,26] Thus, we used a thoracoscopic technique and application on pre-tied ligature loops to collect large ($>3\,cm^2$) lung biopsies from the dorsal aspect of the caudal lung lobe in standing sedated normal horses and horses affected with heaves. The technique was well tolerated with the major complication being ligature slippage (31%). Pneumothorax consistently occurred (21/22 procedures) but typically resolved within 3 weeks. No other major postoperative complications occurred.

*Ligature Slippage*

To counter the relatively non pliable quality of the lung tissue, the caudodorsal border was the preferred site for biopsy because it is a thinner, more malleable lung region. Taking in consideration that the biopsy site was always located at the lung border, we found no statistical difference between complication rate and biopsy site. During collection, when the ligating loop was tightened, entrapped pulmonary tissue was compressed like a sponge creating a well-defined stricture in the lung. A braided multifilament suture loop was chosen to counteract the gliding properties of the visceral pleura and to minimize slippage; however, if too much lung was included in the ligature, the tissue folded on itself. This resulted in a less-than-optimal seat for the ligature and

may have increased the risk of loosening and slippage. Probably the most important factor contributing to ligature slippage was the quantity of tissue distal to the ligature (i.e. between the ligature and cut surface of the lung). Although we attempted to leave $\sim 5$ mm distal to the ligature, in some cases less tissue remained and likely contributed to slippage. Increase precision of transection site was difficult because of lung movement during respiration. It is our impression however, that taking an appropriately-sized biopsy at the thinner margin of the lung and transecting the sample far enough from the ligature (1 cm) should reduce risk of ligature slippage. When slippage occurs, it can usually be corrected by placing another ligating loop around the pulmonary defect. Alternatively, a laparoscopic stapling device[16] can be used if additional ligatures are not effective in closing the defect. Based on our experience with these horses, we would not recommend use of a ligating loop as the sole technique for resection of lung sections $>3\,cm^2$.

*Tissue Sample Size*

Inability to accurately determine the tissue sample size before tightening the ligating loop was considered a disadvantage of this technique. Indeed, 7 biopsies were too small for our purposes; however we did not record how many biopsies were larger than needed. Ideal ligature placement was dependent on our ability to manipulate the lung with the forceps, the thickness of the lung segment being biopsied, and ability to manipulate the loop inside the thorax. Good positioning of the portals was essential, and reduced some of these problems. Nevertheless, even the smaller samples were adequate for histologic analysis.

*Intercostal Hemorrhage*

To introduce a cannula into the thorax, a sharp then a blunt trocar was used. We were careful to direct insertion toward the cranial aspect of the caudal rib to avoid the intercostal vessels. Although rare (2/66 cannula insertions), intercostal hemorrhage did occur and markedly increased surgical time because of the difficulty in placing ligatures through the small incision. Intercostal hemorrhage also reduced clarity of thoracoscopic observation and could have potentially increased infection rate from blood accumulation in the pleural space. Because of anatomic variations in intercostal vascularization, this complication is difficult to prevent but might be minimized by using only a blunt trocar or a smaller size cannula, particularly when discrete palpation of the ICS is difficult.

*Supplemental Oxygen Administration*

The significant decrease in $PaO_2$ that occurred during surgery was most likely attributable to detomidine and exacerbated by pneumothorax.[32] Heaves-affected horses in crisis, also had a significant decrease in $PaO_2$ at baseline and during surgery compared with their baseline and surgical values when in remission. This finding is consistent with respiratory modifications related to the horses' condition,[5] where pulmonary exchange is not optimal and administration of intranasal $O_2$ could improve gas exchange[33] and increase tolerance to surgery. Six of 22 horses, including 4 heaves-affected horses, required intranasal $O_2$ administration (15 L/min) to increase $PaO_2$ and these horses subsequently maintained respiratory rate and $PaO_2$ within normal limits during surgery. Availability of $O_2$ supplementation is recommended when biopsies are performed in horses with compromised respiratory function. Maintaining normal respiratory rate also facilitated instrument manipulation within the thorax.

*Pneumothorax*

Our incidence of pneumothorax (21/22) was higher than previously reported, where 1 out of 10 developed postoperative pneumothorax,[16] despite use of a similar technique. It is possible that the residual space created by the thoracoscope cannula prevented complete reinflation of the lung. Use of a teat cannula might reduce the residual space and improve lung inflation. Although air leakage through the portals was theoretically possible, they were adequately closed[12]; a purse string suture was pre-placed and tied when the cannula was removed, and an adhesive drape was applied. Our purpose in taking serial thoracic radiographs was to detect late leakage from the biopsy sites. Seemingly this only occurred in 2 horses where increased volume of pneumothorax was detected and air was subsequently aspirated. In man and horses, slow aspiration of the pneumothorax is recommended if the condition is severe and of long duration ($>24$ hours) because of the risk of secondary re-expansion pulmonary edema.[34,35] We used a surgical suction unit to rapidly aspirate pneumothorax at the end of the surgery because we speculated that the risk was minimal because of the short surgical duration.

Pneumothorax was not associated with clinical signs and resolved spontaneously within 3 weeks. It has been suggested that pneumothorax be treated conservatively if the horse is not showing signs of respiratory distress.[36] In humans, pneumothorax is reabsorbed passively with 1.25% of lung volume re-expanding each day.[37] We were unable to determine temporal reexpansion volume in our horses.

Radiographic examination has been previously considered an effective method to diagnose pneumothorax.[38] Radiographically, bilateral pneumothorax were detected at 2 hours for 14 procedures; however only 8 bilateral pneumothoraces were identified intraoperatively, so there was a poor correlation between initial radiographic examination and surgical observation. This might be explained by an inability to intraoperatively detect a small quantity of air entrapped in the contralateral thorax and could also be explained by air migration into the contralateral hemithorax in the 2 hours after surgery through small fenestrations in the caudal and the ventral portions of the mediastinum.[38] Interestingly, in our study, unilateral pneumothorax was observed in 5 heaves-affected horses (3 in crisis, 2 in remission) and in 1 control horse postoperatively. Mediastinal fenestrations can become obstructed by exudates in horses with pleural inflammation.[38] It is possible that heaves-affected horses with pleural inflammation may have occluded mediastinal fenestrations, less likely to develop bilateral pneumothorax.

We concluded that thoracoscopic lung biopsy can be performed in horses with impaired respiratory function without increase risk of morbidity compared with normal horses or heaves-affected horses in remission. The relatively high rate of complications associated with the use of the ligating loop should be considered when choosing surgical technique; however, these complications were never life-threatening and could be controlled use of additional ligature loops or more sophisticated instrumentation. Technique limitations included an inability to predetermine biopsy size and difficulty in precision of the transection site to improve ligature security and access to supplemental to intranasal $O_2$ is recommended.

## ACKNOWLEDGMENTS

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# Annexe 3

**Thoracoscopic Lung Biopsies in Heaves-Affected Horses Using a Bipolar Tissue Sealing System**

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Sommaire

Cet article décrit la procédure chirurgicale utilisée pour le prélèvement des biopsies pour l'étude II. La technique, utilisée ici pour la première fois sur des poumons équins, est plus rapide et potentiellement plus sécuritaire que celle utilisée l'étude I. Elle consiste à induire l'hémostase en écrasant les vaisseaux et en émettant de l'énergie bipolaire qui fait fusionner l'élastine et le collagène de la paroi des veines et artères sur quelques millimètres de part et d'autre de la ligne de coupe. La difficulté additionnelle de travailler sur du poumon vient de la présence de bronches qui doivent également être scellées pour éviter le développement d'un pneumothorax sous tension en période postopératoire. La technique s'est avérée être sécuritaire dans la plupart des cas, elle crée des dommages thermiques minimes et elle est très rapide. Les complications observées incluent de l'hypoxémie, un saignement intercostal (n=1) et une fermeture incomplète du site de biopsie (n=2) qui a résulté en un pneumothorax sous tension fatal chez un cheval. En résumé, cette technique semble entraîner des complications moins fréquemment que celle au lasso, mais des complications peuvent être plus difficile à détecter et être potentiellement plus sévères.

**Contribution**
J'ai contribué à l'organisation du transport et de la préparation des chevaux (80%), à la coordination des différentes équipes (75%), à la sédation et au monitoring des chevaux (75%), à l'aspiration des pneumothorax (80%), au suivi postopératoire (90%) et à la rédaction de l'article (10%).

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# Thoracoscopic Lung Biopsies in Heaves-Affected Horses Using a Bipolar Tissue Sealing System

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**Objective:** To validate the use of the LigaSure™ Vessel Sealing System (LVSS) to perform thoracoscopic lung tissue biopsies in heaves-affected horses.
**Study design:** Prospective clinical study.
**Animals:** Heaves-affected horses (n = 12).
**Methods:** Lung biopsies (n = 34) were collected with the LVSS (2–4 biopsies/horse) in horses with and without clinical signs of heaves. Thoracoscope (13th intercostal space [ICS]) and 2 instruments (between the 12–15th ICS) portals were used. Selected clinical and arterial blood gas variables were monitored. Postoperative pneumothorax was evaluated. Depth of thermal injury to the surrounding tissue and representativeness of the biopsies were determined.
**Results:** Mean surgical time was $22.9 \pm 8.0$ minutes. The complication rate was 5.6%, and primarily related to a focal inadequate sealing of the biopsy margin. Five horses in exacerbation required intraoperative intranasal $O_2$. Mean $PaO_2$ was significantly lower in heaves-affected horses with clinical signs compared with those without clinical signs. Postoperative pneumothorax was detected radiographically after 20 of the 34 procedures. One horse with clinical signs of heaves developed a fatal tension pneumothorax 5 days postoperatively despite close radiographic monitoring.
**Conclusion:** Thoracoscopic lung biopsy using LVSS is a rapid and effective technique to harvest peripheral lung tissues from heaves-affected horses. Although the complication rate was tolerable, tension pneumothorax was a potential life-threatening complication because of incomplete lung sealing.
**Clinical Relevance:** LVSS can be used with relative safety to perform thoracoscopic lung biopsy, but close postoperative monitoring is necessary to avoid tension pneumothorax.

Thoracoscopic lung biopsy in horses has previously been performed by several methods. Biopsy forceps allowed harvesting of small lung biopsy samples, which were considered representative of lung tissue.[1] Nevertheless, this technique may require the use of electrosurgery to control pulmonary hemorrhage or air leakage from the biopsy site,[2] although these complications are not reported in horses.[1] Laparoscopic staples have been evaluated in horses with heaves during clinical remission of the disease[3] and this method permitted collection of representative biopsy specimens with a complication rate of 10%. More recently, pretied ligating loops were used in normal and heaves-affected horses both during remission and exacerbation[4] and this technique was judged relatively safe, effective, and more affordable than laparoscopic staples, with a complication rate of 31%. Most commonly, complications were associated with slippage of the ligating loops, which was corrected intraoperatively by application of either another ligating loop or laparoscopic staples.

The LigaSure™ Vessel Sealing System (LVSS) is an electrothermal bipolar sealing system that allows local hemostasis by vessel compression and obliteration through the emission of a bipolar energy. The heat generated induces the fusion of collagen and elastin in vessel walls and subsequent tissue reformation with creation of a permanent seal zone. The system detects the thickness of tissue to be coagulated and automatically calculates the amount of energy required and the appropriate delivery time. An

acoustic signal informs the surgeon when vessel obliteration is complete. Dissection is then possible using the incorporated blade.[5] In people, vessels $\leq 7$ mm in diameter can be safely coagulated with minimal thermal damage to the surrounding tissue extending to a depth of 2 mm.[6,7]

LVSS is routinely used in horses to perform standing or recumbent laparoscopic cryptorchidectomy and ovariectomy[8,9] and removal of large tumoral ovaries ($\leq$30 cm diameter) has been reported.[10,11] Moreover, the use of the LVSS was validated for ligation of the mesenteric vessels in equine small intestinal enterectomy,[12] and was also successfully used to remove the spleen in 1 horse[13,14] and for standing hand-assisted laparoscopic nephrectomy in 4 horses.[15,16]

LVSS use in swine[5,14] resulted in excellent sealing of pulmonary vessels with the bursting strength of the pulmonary seal tissue at 7 days being equal to, or greater than, normal lung tissue. Focal thermal damage extended 3 mm into the surrounding pulmonary parenchyma.[5] Sealing was unpredictable for bronchi with 3–5 mm diameter and negligible if bronchial diameter was $\geq$6 mm. LVSS safely sealed vessels $\leq 7$ mm and lung tissue in thoracoscopic lobectomy in pediatric human patients.[17] When used for lung wedge resection[5] no major complications occurred; 2 of 32 patients had transient air leakage that resolved without further treatment[5] and it was concluded that LVSS was suitable for wedge but not lobe resection where large bronchi are present.

We evaluated the efficacy of LVSS for lung margin biopsy in heaves-affected horses, and quantified thermal damage. We hypothesized that LVSS would be safe and effective for thoracoscopic lung biopsy in heaves-affected horses both in remission and in exacerbation, and that adjacent thermal damage would be minimal. This study was conducted as a part of a study on airway remodeling in equine heaves.

## MATERIALS AND METHODS

### *Case Selection*

Heaves-affected horses (n = 12; approximate age, 13–25 years; mean [$\pm$SD] weight, 499.0 $\pm$ 56.6 kg) were studied. Horses were diagnosed with heaves based on a clinical history of reversible respiratory obstruction and the presence of inflammation in bronchoalveolar lavage fluid when exposed to hay and straw[18]. Three horses had 4 lung biopsies at different periods (3 during remission, 1 during exacerbation), 4 horses had 3 biopsies (2 during remission, 1 during exacerbation) and 5 horses had 2 biopsies (1 during remission, 1 during exacerbation). The protocol of the paired study dictated the number and the schedule of biopsies in each horse.

Thirty-four thoracoscopic surgeries were performed. For horses undergoing 2 biopsies, both hemithoraces were operated at different time points. For horses undergoing 3 or 4 biopsies, one or both hemithoraces were operated twice and the 2 biopsies collected from the same hemithorax were taken at different locations. Horses in remission were considered without clinical signs of heaves based on normal clinical score.[19] All horses were stabled in a dusty environment and were fed hay and bedded on straw to induce clinical exacerbation of the heaves. All horses developed signs of airway obstruction characterized by nasal flaring and increased abdominal effort. These variables were translated into clinical scores as described.[4,19]

### *Surgical Procedure*

Horses were restrained in stocks and sedated with either intravenous (IV) detomidine infusion (loading dose 6 μg/kg; continuous rate infusion 0.8 μg/kg/min for 15 minutes and a half-decrease every 15 minutes thereafter) or detomidine bolus (10 μg/kg IV as needed), while additional analgesia was provided by preoperative IV administration of butorphanol (0.02 mg/kg).[20]

Each hemithorax was clipped and aseptically prepared for surgery. After infiltration of 2% lidocaine (15–20 mL), the skin was incised at the 13th intercostal space (ICS), 5 cm below the *erector spinae* ventral limit. A teat cannula was introduced into the hemithorax through the incision until air aspiration signaling induction of pneumothorax was heard. Then, a 100 mm length, 10 mm diameter reusable cannula (RIWO-ART$^{TM}$ Trocar Sleeve 10 mm, 100 mm length, Richard Wolf Medical Instruments Corp., Milton, ON, Canada) with a sharp trocar was introduced into the incision and through the thoracic wall. Care was taken to move away from the caudal aspect of the cranial rib to avoid the neurovascular bundle. A rigid 33 cm length, 0° laparoscope (Laparoscope MILLENIUM$^{TM}$, 0°, 10 mm, V. Mueller® Product, Cardinal Health, Toronto, ON, Canada) was inserted into the cannula and the hemithorax was explored. Afterwards, 2 instrument portals were created in a similar manner: 1 with 10 mm diameter cannula (RIWO-ART$^{TM}$ Trocar Sleeve 10 mm, 100 mm length, Richard Wolf Medical Instruments) at the 15th ICS for the laparoscopic claw forceps (Claw forceps, 2/3 teeth, 10 mm, 310 mm length, Richard Wolf Medical Instruments), and another with a 12 mm diameter cannula (RIWO-ART$^{TM}$ Trocar Sleeve 12 mm, 100 mm length, Richard Wolf Medical Instruments) between the 12th and the 15th ICS for LVSS (LigaSure$^{TM}$ Vessel Sealing System, 10 mm, 370 mm length, Covidien-Energy Based Devices, Boulder, CO) device. The location of this latter portal was determined relative to the targetted biopsy site and the ability to manipulate the LVSS at that site. Both instrument portals were located $\sim$5 cm ventral to the thoracoscopic portal.

For horses undergoing a subsequent surgery in the same hemithorax, new incisions were performed at least 5 cm away from the previous to avoid scar tissue.

Two surgeons were required for biopsy collection (FR & FD or FR & MM). The caudodorsal tip of the lung was grasped close to the lung margin by the claw forceps and then the LVSS device jaws were opened and engaged on each side of the lung, about 10–20 mm ventral to the claw

forceps. The sealing device was then locked and activated by a foot pedal. When a seal was achieved as indicated by an acoustic signal, the incorporated blade was fired to sever the lung between the sealed margins. Then, the LVSS device was reopened and advanced repeatedly so as to incise around the grasping forceps. The procedure was repeated until the biopsy was released. As the sample was too large to pass through the cannula, the portal skin wound was slightly enlarged with a scalpel blade and the biopsy was exteriorized with the forceps and the cannula through the 15th ICS incision. The biopsy site was carefully evaluated for bleeding or incomplete sealing once the biopsy was released (Fig 1).

The size of the biopsy was subjectively estimated. As multiple analyses were required for paired studies, at least 3 $cm^2$ of lung tissue were required. For histologic analysis only, 1 $cm^2$ was considered sufficient. Thus, if the biopsy was considered too small for the full panel of analyses (<3 $cm^2$) another biopsy was taken and was counted as a separate biopsy procedure for statistical analysis.

During surgery, the presence of a contralateral pneumothorax was assessed through the dorsal mediastinal window. Pneumothorax was considered absent when the contralateral lung was tightly apposed against the mediastinum.

At the end of surgery, the 2 instrument portals were closed with 0 polypropylene in a single layer interrupted pattern in the skin. The lung was reinflated by air aspiration with a surgical suction unit connected to the thoracoscopic cannula.[3,4] A preplaced 0 polypropylene purse string suture was tightened during cannula removal. When contralateral pneumothorax was identified, a teat cannula introduced into the dorsal part of the 13th ICS was used for aspiration to blindly reinflate the lung. Aspiration was considered sufficient when movement of the cannula in the hemithorax was limited by lung counter-pressure. Sterile gauze sponges and an adhesive drape were placed to keep all wounds protected for 24 hours. Surgical time (initial incision to final suture closure), was measured. The number of LVSS device applications as well as complications were recorded.

Procaine penicillin (22,000 U/kg intramuscularly) and phenylbutazone (2.2 mg/kg IV) were administered to each horse at the end of the surgery and continued twice daily for 2 days. Research protocol constraints precluded administration before biopsy removal.

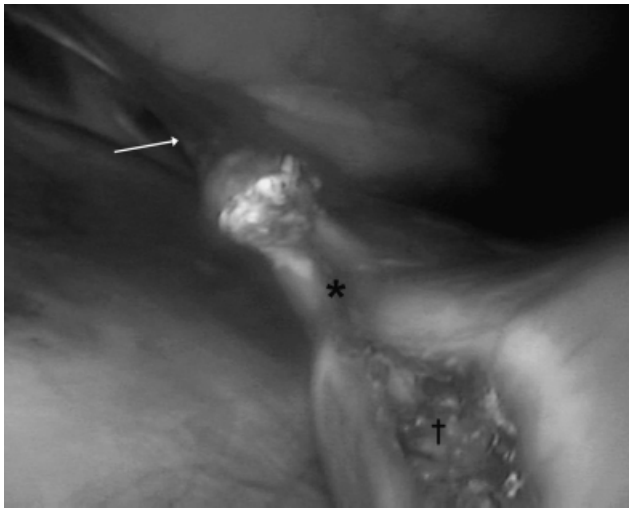


**Figure 1** Biopsy site at the end of the procedure in the right hemithorax. *Complete apposition of the pleural surfaces indicating an adequate seal. †Nonapposition of the pleurae indicating an inadequate seal. Note the ligament of the lung (arrow).

### *Intraoperative Evaluation*

During surgery, heart and respiratory rates were evaluated every 5 minutes. Arterial blood gas analysis ($PaO_2$, $PaCO_2$; Stat 7 Profile Analyzer, Nova Biomedical, Waltham, MA) were performed at least 3 times during surgery: immediately after sedation, 15 minutes after induction of pneumothorax, and at the end of the surgery. Arterial blood samples were taken from the facial artery with a preheparinized tuberculin syringe (1 mL) and immediately processed in a blood gas analyzer. Additional analyses were performed at the anesthetists' discretion (ML).

### *Postoperative Care*

Horses were monitored for the first 24 postoperative hours. Clinical examination was performed at 2 and 12 hours, then twice daily for 2 days, and once daily for 5 days. Horses with postoperative complications were kept under close monitoring until their clinical and blood gas variables normalized, or returned to preoperative values for horses in exacerbation.

### *Radiographic Examination*

A left lateral radiographic examination of the thorax in full expansion was performed preoperatively, and then at 2, 24, and 72 hours postoperatively.[21,22] Only a caudodorsal projection was taken as this view is sufficient to determine presence of pneumothorax (free air in one or both dorsal pleural spaces) and pneumomediastinum (air outlining the aorta).[4,22] Radiographs were assessed by a radiologist (KA) blinded to the biopsy location, time point of radiographic examination and to the horse's condition (remission or exacerbation). The time of maximal pneumothorax was determined by measuring the height of visible gas present in the caudodorsal pleural spaces at their maximal dorsoventral width. The laterality of maximal pneumothorax could be determined in most bilateral cases, as the left pulmonary margin has a more distinct margin than the right one on a left lateral radiograph.[22] If pneumothorax was not resolved after 72 hours, radiographic examination was repeated at a minimum 1-week interval until total resolution was observed.

*Histopathologic Analysis*

Biopsy samples were immediately fixed in 10% neutral-buffered formalin and stored until further processing. Fixed samples were embedded in paraffin, sectioned (4 μm), and stained with hematoxylin–phloxin–eosin–saffron. All sections were analyzed by light microscopy by a pathologist (PH) unaware of horse details.

The depth of thermal damage was estimated using a marker pen and a small ruler, rounded to the nearest value in mm. The internal bronchial circumferences visible in the biopsy specimen were measured using an image analysis software (Image-Pro Plus 6.2, MediaCybernetics, Bethesda, MD) and the mean bronchiolar diameter in each sample was determined by dividing the internal circumference by π (circumference = π × diameter).

*Statistical Analysis*

For analysis, horses were assigned to 2 different groups: heaves-affected horses without clinical signs (Group Ha) and heaves-affected horses with clinical signs (Group Hs). An exact $\chi^2$ test was used to determine the relationship between groups and complication rate, and between presence of intraoperative pneumothorax and groups. A repeated-measures linear model, with time and surgery number as within-subject factors and heaves status as a between-subject factor, was also used to compare vital parameters and arterial blood gas analysis between the 3 different times for the different groups. A repeated-measures linear model, with surgery number as a within-subject factor and heaves status as a between-subject factor, was used to examine differences in surgery time between both groups. The level of significance was set at $P < .05$ for all analyses.

## RESULTS

*Surgical Procedure*

Mean ± SD surgical time was 23.0 ± 8.1 minutes (range, 13–46 minutes) with no identified group effect on surgical time. The first 17 procedures were performed with detomidine infusion; however, sedation had a tendency to be too deep or to last too long and therefore, the last 17 procedures were performed using a detomidine bolus repeated as needed. For 15 procedures only 1 bolus was required, whereas a 2nd bolus was administered to 2 horses.

Two biopsies were subjectively considered too small (<3 $cm^2$) for the analyses required for the research protocol. However, they were considered large enough for histologic analysis (≥1 $cm^2$). A 2nd biopsy was taken in the same manner, resulting in a total of 36 biopsies.

The LVSS instrumental portal had to be repositioned during 11 procedures as its initial location did not allow the instrument to easily reach the biopsy site. A 4th portal was created in a more appropriate location, usually 1 ICS more caudally. The mean number of device applications was 5.3 ± 1.5 (range, 3–10 device application) per biopsy. There was no group effect on the number of device applications.

Intraoperative complications occurred in 3 of 36 procedures, including 1 case of intercostal bleeding and 2 inadequate seals at the biopsy margin. The intercostal bleeding occurred at the insertion site of the 10 mm diameter cannula in a horse with clinical signs of heaves, and was resolved by opening the incision, localizing the vessel and applying ligatures using an absorbable suture. Focal inadequate sealing of the biopsy margin was observed during the procedure in 1 horse with, and 1 horse without, clinical signs of heaves (Fig 1), resulting in a LVSS-specific complication rate of 5.6%. Based on our previous experience with small sealing defects with other techniques, and because these focal areas were small we let the defects heal by 2nd intention. These 2 horses were monitored closely, clinically and radiographically, during the postoperative period. No group effect on complication rate was identified.

During surgery, 2 horses (1 Ha, 1 Hs) developed bilateral pneumothorax identified by the presence of air in the contralateral hemithorax. The contralateral hemithorax of these horses were blindly reinflated at the end of the surgery.

For 7 cases, (3 that had 4 biopsies and 4 that had 3 biopsies) a 2nd thoracoscopy was performed on a previously operated hemithorax, allowing evaluation of the scar from the previous surgery. This reevaluation was performed at least 10 months after initial surgery. For all of them, the previous biopsy site was clearly visible (white scar tissue with clear demarcation line with normal pulmonary tissue) but no major macroscopic abnormalities of the surrounding lung tissue were detected such as exuberant scarring. Five randomly chosen horses were euthanatized at least 4 months after the last biopsy and the lung was evaluated histologically. Macroscopically a large surface (5 × 8 cm) of modified tissue was observed in 2 of them surrounding the lung biopsy sites. This modified tissue was compatible with an excessive scar tissue (Fig 2A).

*Intraoperative Variables*

Intranasal $O_2$ (10 L/min) was administered intraoperatively to 4 Hs horses based on $PaO_2 < 60$ mmHg or presence of labored breathing. Another Hs horse was treated with intranasal $O_2$ preoperatively because of a severe respiratory distress. Surgery was relatively well tolerated by these 5 Hs horses with $O_2$ supplementation. As modification of $PaO_2$ was induced by oxygen therapy, these horses were excluded from blood gas statistical analysis; however, mean $PaO_2$ and respiratory rate for these horses during oxygen therapy were 68.6 (± 7.7) mmHg and 20 (± 9) breaths/min respectively.

Thus, when considering only horses not supplemented with inhaled $O_2$, there was a significant ($P = .0009$) decrease in $PaO_2$ during surgery in horses in clinical exacerbation (n = 7) compared with horses in clinical remission

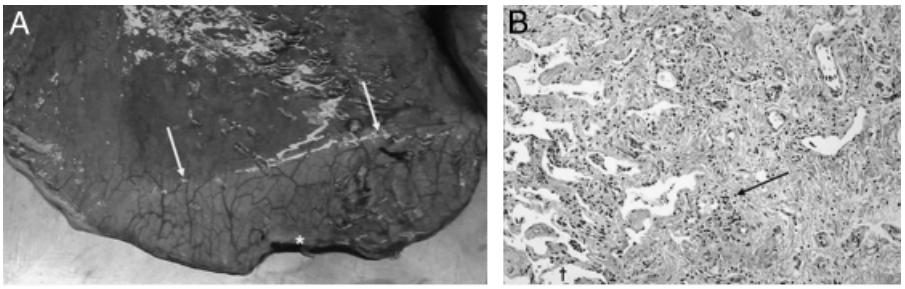


**Figure 2** Left lung. (A) modified tissue compatible with excessive scar tissue (white arrows) surrounding the previous biopsy site (*). Microscopic hematoxylin–phloxin–eosin–saffron staining ( × 40) of the corresponding lung (B) showing the extensive replacement of the lung parenchyma by mature fibrous connective tissue with distortion of the alveoli and terminal bronchiolar lumens (†). There are also scattered lymphocytes and plasma cells in the fibrous tissue (black arrow).

(n = 16). There was no effect of the case status or the time on respiratory rate, heart rate, and $PaCO_2$ (Fig 3).

### *Radiographic Examination*

Postoperative radiographic examination revealed no pneumothorax in 10 horses (5 Ha, 5 Hs), unilateral pneumothorax in 12 (9 Ha, 3 Hs) and bilateral pneumothorax in 10 (8 Ha, 2 Hs), including 2 Hs horses with no pneumothorax in the early postoperative period. These 2 horses developed a bilateral pneumothorax within 72 hours postoperatively. Postoperative radiographic evaluation of the thorax was not performed in 2 horses because of a technical problem. There was no effect of the horse's status on the occurrence of the uni or bilateral pneumothorax. Pneumothorax resolved spontaneously within 3 weeks postoperatively for 17 of 22 cases. One Hs horse with inadequate sealing of the biopsy site developed a fatal tension pneumothorax 5 days postoperatively. For this horse, maximum pneumothorax was identified 72 hours postoperatively and was subjectively not worse compared with other cases.

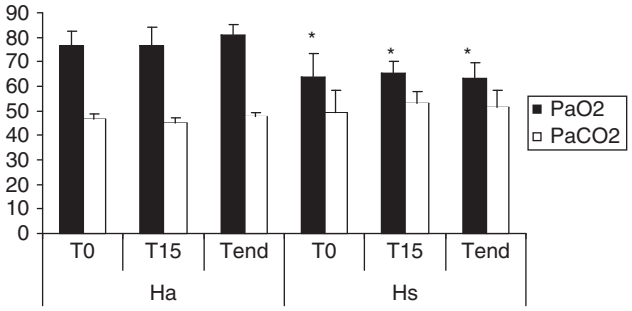


**Figure 3** Mean (± SD) $PaO_2$ and $PaCO_2$ mmHg at the beginning of surgery before induction of pneumothorax (T0), 15 minutes after induction of pneumothorax (T15) and at the end of surgery (Tend) for heaves-affected horses with clinical signs of heaves (Ha) and without clinical signs of heaves (Hs). Horses receiving intranasal oxygen where excluded. *Significant difference ($P < .05$) between Ha and Hs groups.

Maximum pneumothorax was recorded 2 hours postoperatively for 7 horses (4 Ha, 3 Hs), 24 hours postoperatively for 9 horses (8 Ha, 1 Hs) and 72 hours postoperatively for 6 horses (5 Ha, 1 Hs). The side with maximum pneumothorax could not be determined in 4 horses (1 Ha, 3 Hs). The side with maximum pneumothorax matched the operated hemithorax for 11 horses (7 Ha, 4 Hs) and was opposite for the 7 other horses (5 Ha, 2 Hs).

### *Postoperative Care*

After surgery, 18 horses (10 Ha, 8 Hs) developed focal subcutaneous emphysema and 1 Hs horse developed extensive subcutaneous emphysema up to the neck; however, emphysema was not clinically relevant in any cases and resolved spontaneously within a few days.

As mentioned, 1 Hs developed fatal tension pneumothorax 5 days postoperatively. This horse had a small focal inadequate sealing of the biopsy site that was left to heal by 2nd intention. Close monitoring during the postoperative period revealed no obvious changes in the clinical status of this horse. Bilateral pneumothorax was present at 2 hours postoperatively and was maximal at 72 hours. Two additional radiographic examinations were performed in the following days because the horse's condition remained severe and revealed no change in the degree of pneumothorax. Furthermore, the pneumothorax was not worse than the others noted in horses with adequate sealing. We did not attempt to reaspirate the pneumothorax because no deterioration of the clinical and radiographic signs or blood gas variables was observed. While the cause of death was not clearly determined, the necropsy suggested presence of a tension pneumothorax. A defect in the pleural seal was present and its size was similar to that observed at the end of the surgery.

Another horse with a focally inadequate seal developed pneumothorax postoperatively that was similar to those with an adequate seal and this resolved in similar time to pneumothorax in horses with adequate seals.

One horse developed mild fever (39.3°C), the cause of which was not identified and it resolved spontaneously within 2 days. Another horse developed fever (39.5°C),

believed to be of viral origin, 2 weeks postoperatively; the fever was transient (3 days) and resolved uneventfully.

*Histopathologic Analysis*

Specimens contained multiple vessels and airways, pulmonary architecture was well preserved, and multiple inflated alveoli were present in all the biopsies. Most lesions consisted of pulmonary fibrosis and epithelial hyperplasia associated with neutrophilic inflammation within the airway lumen, findings representative of heaves (Fig 2B). A distance of $2.48 \pm 1.06$ mm (range, 1–4 mm) from the biopsy margins was unreadable because of thermal damage (Fig 4). Slight thermal artifact that did not prevent analysis involved a deeper region (mean depth, $1.50 \pm 0.60$ mm; range, 0–2.5 mm) and was present in one-third of samples. In total, thermal damage extended to a depth of $3.38 \pm 1.54$ (range, 1–6.5) mm. Mean bronchiole diameter was $370.74 \pm 247.79$ (range, 63.25–1845.38) μm.

## DISCUSSION

We report use of LVSS to perform thoracoscopic lung biopsy in standing heaves-affected horses, both in remission and during clinical exacerbation. Although its use has been reported in swine,[5,14] and humans[5,17] we are not aware of previous reports of its use in horses for lung surgery. The procedure was well tolerated by horses even when in exacerbation although 5 of them required intranasal oxygen administration intraoperatively, as reported when pretied ligating loops were used for lung biosy.[4] A focal inadequate pleural seal occurred in 2 of 36 procedures; it spontaneously resolved in 1 horse, but was fatal for 1 horse at 5 days. Transient postoperative pneumothorax was also identified after 20 procedures. Histologically, the depth of thermal injury surrounding the biopsy margin was minimal relative to biopsy size.

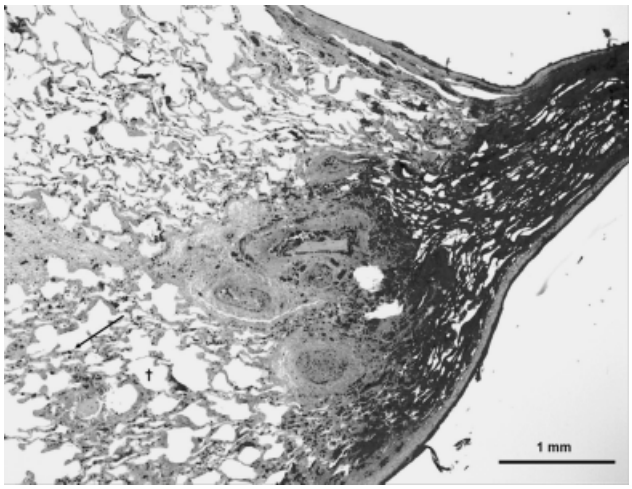


**Figure 4** Microscopic hematoxylin–phloxin–eosin–saffron staining ($\times 40$) of a biopsy sample showing the clear demarcation between the normal parenchyma with alveoli lumen (†) and neutrophilic infiltration (black arrow), and the thermal damage with loss of cell and alveoli definition.

Mean surgical time ($23 \pm 8$ minutes) compared favorably with the use of laparoscopic staples ($28 \pm 5$ minutes),[3] and was shorter than use of pretied ligating loops ($42 \pm 17$ minutes).[4] This shorter surgical time could be explained by easier manipulation of the LVSS device compared with the ligating loop around the forceps grasping the lung. The seal with LVSS was rapidly obtained, and the low number of applications to the lung also contributed to the shorter duration of the surgery. Adequate location of the instrument portal was critical to allow an adequate approach to the biopsy site; a 4th instrumental portal was necessary for 11 biopsies. Considering this, we recommended creating the instrument portal after thoracic exploration, when the lung is completely deflated. The use of a device with longer jaws could further help decrease surgical time. The device we used was straight and the development of an angulated or reticulated LVSS device as for the laparoscopic staples, would improve its versatility.

The complication rate directly related to use of the LVSS was relatively low (5.6%). Two horses had focal inadequate seal of the biopsy margin seen when evaluating the biopsy site after the biopsy was released. This was probably caused by the large thickness of tissue to seal. The energy delivered by the LVSS is directly dependent of the amount of tissue between its jaws and is determined by a difference of impedance. When the jaws are applied to the lung, the jaws crush the lung and reduce tissue thickness. Nevertheless, when the jaws are reopened, the lung reexpands. This could have increased tension on the previously sealed biopsy margin. During surgery, the seal was complete immediately after the release of the jaws, and the reopening occurred in the following seconds. Thus, reevaluation of biopsy site is advised at the end of the procedure to identify potential reopening of the biopsy margin. Late reopening in the 1st postoperative day because of thermal necrosis is also possible until scar tissue secures the seal.

The depth of the thermal injury was 3 mm after LVSS use in people.[5] We found a distance of 2.5 mm from the biopsy margin was unreadable for histologic analysis, and a further 1.5 mm depth revealed some minor thermal damage that did not prevent histopathologic analysis. Thermal damage was therefore deeper than previously reported in both people and swine. This was likely related to greater thickness of sealed tissue in horses than in other species. More energy was required to seal the tissue, leading to more energy diffusing from the jaws to the surrounding tissue. This margin should be taken into account to obtain diagnostic-quality biopsies.

Pneumothorax, hemothorax, or respiratory distress[2,23] are possible complications associated with lung biopsies, whether performed percutaneously using a needle[24,25] or thoracoscopically using biopsy forceps,[1,2] although not reported in horses using the latter technique.[1] In the present study, pneumothorax was detected postoperatively in 22

of 34 cases. Although a small area of the visceral pleurae was left unsealed at the end of the procedure in 2 cases, this cannot explain all the pneumothoraces observed. Incomplete aspiration at the end of the procedure may explain some of the pneumothoraces detected 2 hours after surgery. An air-leak form the skin wounds during the first 48 hours cannot be excluded. Finally it is possible that either our visual assessment overestimated the sealing of the surgical site in some cases or that it reopened post surgically because of thermal necrosis of the surrounding pleurae. While the residual pneumothorax resolved spontaneously in most cases, 1 horse suddenly died 5 days postoperatively, presumably from a tension pneumothorax despite close clinical and radiographic monitoring. As the pneumothorax was moderate and did not further increase post surgically on serial radiographs, the breathing difficulties were believed associated with the primary respiratory problem, and treatments were administered accordingly. However, it is likely that lung hyperinflation may have prevented lung collapse and therefore appropriate appreciation of the pneumothorax severity based on radiographic evaluation. Placement of a thoracostomy tube attached to a Heimlich valve would be a safe procedure to prevent development of a life-threatening tension pneumothorax for any horse which is deteriorating or not quickly improving after surgery.[26]

Only a lateral caudodorsal radiographic projection was evaluated to assess the severity of pneumothorax. Based on previous studies,[3,4] post thoracoscopy complications were pneumothorax or pneumomediastinum. Pneumothorax is better detected in the caudodorsal part of the thorax where the air accumulates in a standing horse.[4,22] Pneumomediastinum was diagnosed by the presence of air outlining the aorta, which was also seen on the caudodorsal view.[4,22] Thus, performing this single view seemed sufficient to diagnose potential major complications while limiting radiation exposure. Ultrasonography may be an alternative to diagnose pneumothorax or pleural effusion. A full-thoracic radiographic examination is nonetheless recommended in horses showing clinical signs of complications other than pneumothorax (ie bronchopneumonia, pulmonary abscess).

At least 10 months after the 1st surgery, macroscopic evaluation of biopsy sites revealed a large amount of fibrous tissue at the initial biopsy site in 2 horses (Fig 2A). Analysis performed on these 2 cases ruled out the possibility of a tumoral response. An excessive response of the body to the thermal injury has been suggested. Excessive diffusion of thermal energy may have lead to extensive necrosis and scarring, possibly because of an increase in delivered energy associated with tissue thickness. However, it was not possible to quantify the energy delivered. A focal infection could not be excluded, although no external/clinical signs of this were observed. Because all other horses had an expected amount of scar tissue, this exuberant reaction at the biopsy site is most likely to be an individual response.

We concluded that use of the LVSS to perform thoracoscopic lung biopsy in heaves-affected horses was a rapid and relatively safe technique. Oxygen insufflation was required in some cases during the procedure. The complication rate was lower than in other described techniques[1,3,4]; however, clinical and radiographic monitoring post surgically is required because of the potential development of tension pneumothorax, as occurred in 1 horse. The quality of the lung sample was histologically appropriate, except for a band of thermal damage extending from the biopsy margin for $2.5 \pm 1.5$ mm.

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# Annexe 4

**Profiling of Differentially Expressed Genes using Suppression Subtractive Hybridization in an Equine Model of Chronic Asthma**

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Sommaire

Cet article décrit l'expression génique du tissu pulmonaire périphérique de chevaux atteints du souffle en utilisant une technique qui permet de détecter les gènes surexprimés chez les chevaux symptomatiques. La technique d'hybridation suppressive soustractive (SSH) permet en effet de « soustraire » les gènes exprimés chez les chevaux sains et les chevaux atteints asymptomatiques, des gènes exprimés par ces mêmes chevaux en période d'exacerbation. Des 950 gènes surexprimés, 224 ont été séquencés. Le pattern d'expression a été confirmé pour 15 des 22 gènes testés par PCR quantitatif. Certains gènes identifiés sont intéressants pour leur rôle dans l'inflammation pulmonaire et d'autres gènes ont possiblement un rôle à jouer dans le remodelage du muscle lisse. Une quinzaine de gènes sont associés à la contraction musculaire ou au remodelage du muscle lisse. Dans le contexte du remodelage du muscle lisse péribronchique, ces résultats pourront servir de point de départ pour l'étude de l'expression génique spécifique au muscle isolé par microdissection.

**Contribution**

J'ai contribué à l'élaboration des protocoles de chirurgie, aux soins préopératoires et à la manipulation des tissus afin de minimiser les effets sur l'expression génique (20%), à l'analyse des données (20%) et à la rédaction de l'article (10%).

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# Profiling of Differentially Expressed Genes Using Suppression Subtractive Hybridization in an Equine Model of Chronic Asthma

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## Abstract

***Background:*** Gene expression analyses are used to investigate signaling pathways involved in diseases. In asthma, they have been primarily derived from the analysis of bronchial biopsies harvested from mild to moderate asthmatic subjects and controls. Due to ethical considerations, there is currently limited information on the transcriptome profile of the peripheral lung tissues in asthma.

***Objective:*** To identify genes contributing to chronic inflammation and remodeling in the peripheral lung tissue of horses with heaves, a naturally occurring asthma-like condition.

***Methods:*** Eleven adult horses (6 heaves-affected and 5 controls) were studied while horses with heaves were in clinical remission (Pasture), and during disease exacerbation induced by a 30-day natural antigen challenge during stabling (Challenge). Large peripheral lung biopsies were obtained by thoracoscopy at both time points. Using suppression subtractive hybridization (SSH), lung cDNAs of controls (Pasture and Challenge) and asymptomatic heaves-affected horses (Pasture) were subtracted from cDNAs of horses with heaves in clinical exacerbation (Challenge). The differential expression of selected genes of interest was confirmed using quantitative PCR assay.

***Results:*** Horses with heaves, but not controls, developed airway obstruction when challenged. Nine hundred and fifty cDNA clones isolated from the subtracted library were screened by dot blot array and 224 of those showing the most marked expression differences were sequenced. The gene expression pattern was confirmed by quantitative PCR in 15 of 22 selected genes. Novel genes and genes with an already defined function in asthma were identified in the subtracted cDNA library. Genes of particular interest associated with asthmatic airway inflammation and remodeling included those related to PPP3CB/NFAT, RhoA, and LTB4/GPR44 signaling pathways.

***Conclusions:*** Pathways representing new possible targets for anti-inflammatory and anti-remodeling therapies for asthma were identified. The findings of genes previously associated with asthma validate this equine model for gene expression studies.

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*[redacted]

## Introduction

Inflammation and remodeling of the airway wall are characteristic features of asthma. The term ''airway remodeling'' in bronchial asthma is used to describe the structural changes that occur in conjunction with, or because of, chronic inflammation. A consequence of asthmatic airway remodeling is incompletely reversible, or even irreversible airway obstruction, bronchial hyperresponsiveness, and an accelerated decline in lung function [1]. Remodeling processes in asthma result from highly complex, and poorly defined interactions between inflammatory and resident structural cells [2]. Therefore, the identification of the molecular pathways involved in the crosstalk between these cells is a prerequisite for the development of novel therapy to control airway remodeling.

Expression profile studies allow the discovery of transcripts correlated to disease phenotype and to generate hypotheses regarding genes and pathways underlying these phenotypic changes. Gene expression studies using human lung tissues have been primarily derived from the analysis of bronchial biopsies harvested from mild to moderate asthmatic subjects and controls [3]. These studies have identified candidate genes and pathways

related to asthma pathogenesis. There is however limited information on the transcriptome profile of the peripheral lung tissues where remodeling predominantly occurs in non-fatal asthma [4,5]. Using rodent models of asthma, microarrays analyses of whole lung tissues have been used to reveal the complex signaling pathways associated with the initiation of the asthmatic response. However, mice have important differences in the anatomy of the lungs compared to humans, including the relative paucity of airway smooth muscle [6]. Furthermore, sensitization to multiple antigens and recurrent challenges over many years do not occur, thus making the immune response and the crosstalk between structural cells potentially less complex than in people.

Studies of comparative pulmonary morphology show that the horse's lung closely resembles the human lung [7,8] and their lifespan (30–35 years) is closer to human than small rodents. Also, 10 to 20% of horses develop a condition called heaves that shares many features of "extrinsic" human asthma, including lower airway inflammation, reversible airflow obstruction, and bronchial hyperresponsiveness [9,10,11]. Heaves develop spontaneously in susceptible horses and, similarly to asthma, is associated with increased airway smooth muscle mass, goblet cell hyperplasia, and epithelial detachment and regeneration [12,13,14,15]. The horses size and temperament also allow for multiple sampling from the same animal to compare gene expression of the lung tissue under conditions of disease exacerbation and remission. Thus, equine heaves is an appealing model to study the complex inflammation-induced remodeling processes present in chronic asthma.

Suppression subtractive hybridization technique (SSH) is a highly sensitive PCR-based cDNA subtraction method [16] used to identify differentially expressed genes, including genes of relatively low abundances. It selectively amplifies differentially expressed cDNA fragments while suppressing nontarget cDNA amplification. SSH provides an approximately 1000-fold enrichment of low copy number genes related to defined phenotypes [17]. Compared to microarray analysis, SSH is more sensitive, sequence independent and yields relatively few false positive [18]. The goal of this study was to document the transcriptome associated with chronic asthmatic inflammation and tissue remodeling. We use SSH to subtract the lung transcriptome obtained from heaves-affected horses during clinical remission as well as from control horses with or without antigen exposure from lung cDNAs of horses with heaves after a 30-day antigen challenge.

## Materials and Methods

### Experimental animal model, tissue collection, and RNA isolation

Eleven horses (450–550 kg) including 9 mares and 2 geldings were studied. Six horses with heaves (mean ± SD, 16.8±2.14 years of age) had a history (mean duration 6.4±2.9 years) of recurrent episodes of airway obstruction upon hay exposure, abnormal respiratory mechanic measurements, and increased neutrophils in bronchoalveolar (BAL) fluid. Control horses (n = 5, 13.8±2.39 years of age) had no history of respiratory diseases. Detailed description of the animals, their airway function and lung inflammation have been reported elsewhere [15]. All experimental procedures were performed in accordance with the Canadian Council for Animal Care guidelines and were approved by the Animal Care Committee of the Faculty of Veterinary Medicine of the Université de Montréal (06-Rech-1324).

All animals were kept together in a low antigenic environment (Pasture) for >3 months prior to the baseline measurements and were then stabled in box stalls for 30 days where they were exposed to hay and barn dust (Challenge). Large peripheral lung biopsies were obtained at baseline and after the 30-day challenge by thoracoscopy as reported previously [19]. Biopsies were snap frozen in liquid nitrogen within 3 minutes and stored for a maximum of 5 months at −80°C until RNA extraction.

Total RNA was isolated as previously described [20]. The concentration of total RNA was quantified by measuring optical density at 260 nm using a spectrophotometer (NanoDrop ND-1000, NanoDrop products, Wilmington, DE, USA). The Agilent 2100 bioanalyzer (Agilent Technologies, Wilmington, DE, USA) was used to calculate the RNA integrity number (RIN).

### Suppression subtractive hybridization

SSH was used to compare gene expression in lung tissues of symptomatic heaves-affected horses during challenge (SH) versus heaves-affected horses at baseline and controls at both time points (all regrouped as "Ctls"). Identical amounts of total RNA from each horse were pooled within SH (n = 6) and Ctls (n = 16) groups to decrease inter-animal variation (Figure 1.B). The SSH procedures were performed as previously reported [21] and are illustrated in figure 1.A and C. In brief, double-stranded cDNA were generated using the SMART PCR cDNA Synthesis Kit for both SH and Ctls samples according to the manufacturer's instruction (user manual PT3041-1, Clontech Laboratories, Inc., Mountain View, CA, USA). One µg of total RNA from each pooled groups were reverse transcribed in a total volume of 10 ul with two primers (3′ SMART$^{TM}$ CDS Primer II A and SMART II$^{TM}$ A Oligonucleotide) and PowerScript reverse transcriptase (Clontech Laboratories, Inc., Mountain View, CA, USA), with the addition of 42 ng of T4 gene 32 protein (Roche Applied Science, Laval, QC, CA) to produce first cDNA strand. The resulting cDNA pools were diluted to 50 µl in TE buffer (10 mM Tris pH 8, 1 mM EDTA). Double-stranded cDNA were obtained via PCR amplification of 19 cycles with 5′PCR Primer II A using Advantage II DNA polymerase (Clontech Laboratories, Inc., Mountain View, CA, USA).

Subtracted forward (SH-Ctls) and reverse (Ctls-SH) reactions were generated by subtracting Ctls cDNAs from SH cDNAs and SH cDNAs from Ctls cDNAs, respectively, using the PCR-select cDNA Subtraction Kit (user manual PT1117-1, Clontech Laboratories, Inc., Mountain View, CA, USA). To perform the subtraction reactions, SH and Ctls cDNAs were digested with RsaI to obtain shorter, blunt-ended molecules suitable for adaptor ligation and optimal for subtractive hybridization.

Subtraction efficiency was evaluated by comparing the abundance of known genes in subtracted and unsubtracted cDNAs population after different cycles of PCR using Advantage II DNA polymerase (Clontech Laboratories, Inc., Mountain View, CA, USA). Equine gene specific primers were designed for two genes; one that is constitutively expressed, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*); and lipocalin 2 (*LCN2*), a gene known for its association with lung diseases [22] (Table S1). The subtraction efficiency was indicated by the difference in the number of cycles needed to generate equal amplification of the corresponding PCR product before and after subtraction for these two genes.

### Cloning of subtracted cDNAs

The purified (QIAquick PCR Purification kit, Qiagen, Toronto, ON, CA) subtracted (SH-Ctls) cDNAs were cloned into the pDrive-cloning vector (Qiagen, Toronto, ON, CA). Ligation products were then used to transform competent cells (DH5α, Invitrogen, Carlsbad, CA, USA), which were spread onto S-Gal®LB agar

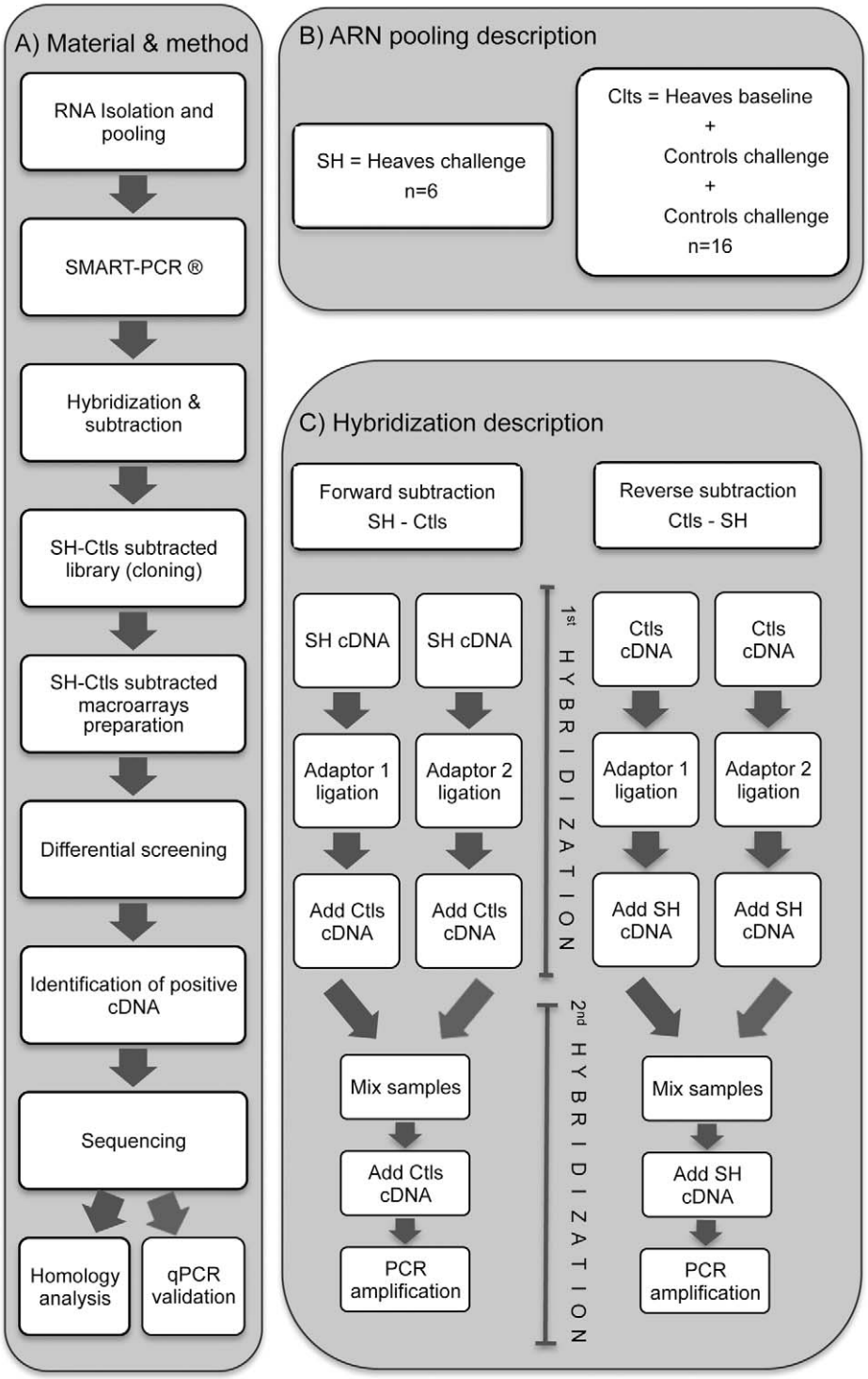


**Figure 1. Methodology for SSH.** Schematic representation of the different steps described in Material and Methods (A), the sample pooling (n corresponds to the number of samples) (B), and the different hybridization steps performed with the SSH technique (C).
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blend plate (Sigma-Aldrich Canada Ltd, Oakville, ON, CA) supplemented with kanamycin (40 µg/ml). Individual colonies (950) were transferred into ten 96-well plates containing LB freezing media (8.8% glycerol, 55 mM $K_2HPO_4$, 1 mM $MgSO_4$, 26 mM $KH_2PO_4$, 15 mM $NH_4(SO_4)$) and incubated overnight to construct the SH-Ctls subtracted library.

## Differential hybridization screening

SH-Ctls subtracted macroarrays were established for differential screening, as previously described [21]. Briefly, PCR amplification was performed on the insert of each cDNA clone from the SH-Ctls subtracted library plates using AmpliTaq DNA Polymerase (Applied Biosystems, Foster City, CA, USA), with PCR-nested

primers 1 and 2R (Clontech Laboratories, Inc., Mountain View, CA, USA) for 27 cycles. PCR products from each insert were denatured in 0.3 M NaOH colored with 5% bromophenol blue and vacuum-transferred onto nylon membranes (Hybond-N+, Amersham Pharmacia Biotech, Pointe-Claire, QC, CA) using a 96-well dot-blot apparatus and cross-linked to the membrane with UV light (150 mJ, GS Gene Linker, Bio-Rad, Mississauga, ON, CA). Positive control cDNA (*LCN2*) was also transferred on each membrane. For each SH-Ctls subtracted library plates, four identical cDNA macroarray membranes were generated.

Probes for the differential screening of macroarray membranes were obtained from unsubtracted (SH and Ctls) and subtracted (SH-Ctls and Ctls-SH) complex cDNA populations via secondary nested PCR amplification (PCR-select cDNA Subtraction Kit, Clontech Laboratories, Inc., Mountain View, CA, USA). Probes were then purified (QIAquick PCR Purification kit, Qiagen, Toronto, ON, CA), digested with AfaI, SmaI and EagI to remove adaptors and purified again (QIAquick PCR Purification kit, Qiagen, Toronto, ON, CA). One hundred nanograms of the cDNA probes were labeled by random priming with $\alpha^{32}$P[dCTP] (Megaprime DNA labeling System, GE Healthcare, Buckinghamshire, UK) as described previously [21]. Radio-labeled cDNA probes were purified (QIAquick Nucleotide Removal Kit, Qiagen, Toronto, ON, CA) and quantified with a liquid scintillation analyzer (Tri-Carb 2100TR, Packard BioScience Compagny, Meriden, CT, USA).

Each membrane was individually hybridized and washed as describe previously [21]. SH-Ctls macroarray membrane replicates were hybridized with identical amounts (cpm) of specific heat denatured cDNA probe (SH-Ctls, Ctls-SH, SH or Ctls). Washed membranes were exposed to a phosphor screen for 4 hours and the images were digitized (Storm 840, GE Healthcare, Buckinghamshire, UK). cDNA clones with different hybridization pattern were characterized using DNA sequencing and gene expression analysis.

### DNA sequencing and sequence analysis

The differentially expressed cDNA clones were amplified by PCR from the PCR products generated initially for the macroarrays using the Advantage II DNA polymerase (Clontech Laboratories, Inc., Mountain View, CA, USA) and the PCR-nested primers 1 and 2R. Amplicons were analyzed on 1.5% agarose gel with ethidium bromide to detect multiple PCR fragments. Single band amplicons were gel extracted (QIAquick Gel Extraction kit, Qiagen, Toronto, ON, CA) and sequenced via the dideoxy method (Big Dye Terminator 3.0, ABI Prism, Applied Biosystem, Foster City, CA, USA) by Génome Québec using the PCR-nested primers 1 or 2R. Sequencing reactions were analyzed with an ABI Prism 310 sequencer (Applied Biosystem, Foster City, CA, USA). Nucleic acid sequences with at least 100 bp were aligned against GenBank database (NR and EST) using the Basic Local Alignment Search Tool (BLAST). The maximum expected (E) value accepted to be considered homologous was $e^{-30}$. Every match with higher E value score was aligned against the horse genome at the UCSC Genome Browser database using BLAT (BLAST-like alignment tool; http://genome.ucsc.edu/). Sequences were classified into two groups: I) genes with known sequence and function and II) genes with characterized sequence but unknown function. The genes from the 1st group were further classified through biological function categories using functional mapping tools (GeneOntology, http://www.geneontology.org/) and compared to available literature related to human asthma and other animal models. This classification allowed us to identify biological pathways, gene families or biological functions likely to be relevant to airway remodeling and, thus, to select candidate biomarkers possibly associated with asthma.

### Validation step: Gene expression analysis

Quantitative PCR (qPCR) was used to validate the differential gene expression of 22 positive cDNA clones from the SH-Ctls library. First strand cDNA were generated using the SMART PCR cDNA Synthesis Kit (Clontech Laboratories, Inc., Mountain View, CA, USA) and the SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) for each individual horse at baseline and after challenge as described above. When possible, equine gene-specific primers (Table S1) were designed to span at least two intron-exon boundaries for the discrimination of contaminant genomic DNA. The absence of nonspecific products was confirmed by the analysis of the melting point curves and by electrophoresis in 1.5% agarose gels. All concentrations of target gene cDNA were calculated relatively to their respective standard curves. One microliter of cDNA template was added to the Quantitec SYBR®Green PCR Kit master mix (Qiagen, Toronto, ON, CA). qPCR reactions were performed in a volume of 20 µl using Rotor-Gene RG-3000 (Corbett Research, Sydney, AS) and qPCR conditions were similar for all primer sets (0.5 µM, final concentration): denaturation 95°C for 10 min, cycling 95°C for 15 sec, 55°C for 25 sec and 72°C for 25 sec for a maximum of 40 cycles. Each reaction was run in duplicate with the appropriate negative control. Reference gene expression was evaluated using different analysis softwares: NormFinder [23], GeNorm$^{PLUS}$ [24] and Rest 2009 [25].

### Statistical analysis

Data are presented as mean ± SD. Differences between groups were compared at each time point using Wilcoxon tests and differences within groups were evaluated using Mann-Whitney test. Unilateral tests were used to compare values with those of asthmatic horses after 30 days of antigen challenge because SSH technique predicted the direction of the effect. Bilateral tests were used for all other analyses. $P<0.05$ was considered significant.

## Results

### Experimental animal model

All horses had normal lung function prior to challenge, while only horses with heaves developed clinical signs of airway obstruction and persistent airway inflammation after antigen challenge (see [15] for detailed description of lung function and BAL fluid cytology in these animals).

### Identification of differentially expressed genes

The quality of the RNA samples was confirmed by high RIN values (8.47±0.58 SD) and electropherogram analysis. Subtraction efficiency was evaluated using standard PCR for two genes; *GAPDH* and *LCN2*. *GAPDH* PCR products were detectable after only 18 cycles in the SH unsubtracted sample, whereas 10 more cycles were required to detect the PCR fragment in the SH-Ctls subtracted sample (Figure 2.A), a 40 fold reduction. *LCN2* PCR products were detectable after 20 cycles in both subtracted SH-Ctls and unsubtracted SH samples, but the difference in intensity between the two signals indicates enrichment (Figure 2.B). Conversely, the *LCN2* PCR products were detected after 20 cycles in the unsubtracted Ctls sample and after 10 more cycles (40 fold increase) in the reverse subtracted Ctls-SH sample.

Differential hybridization screening was performed using macroarrays in order to isolate genes implicated in heaves exacerbation from the 950 randomly selected clones. Differentially

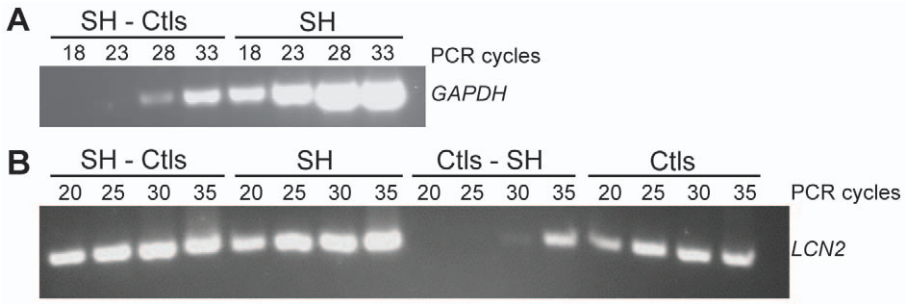


**Figure 2. Evaluation of subtraction efficiency.** A: Reduction of *GAPDH* cDNA following subtraction in the SH-Ctls sample. PCR was performed on SH-Ctls subtracted and SH unsubtracted samples. *GAPDH* PCR products (760 pb) were detectable 10 cycles earlier in the unsubtracted sample (18 cycles) than in the subtracted sample (28 cycles). B: Enrichment of *LCN2* cDNA following subtraction in the SH-Ctls sample. PCR was performed on SH-Ctls and Ctls-SH subtracted samples as well as SH and Ctls unsubtracted samples. *LCN2* PCR products (210 pb) were detected after 20 cycles for both SH unsubtracted and SH-Ctls subtracted samples, the difference in the intensity of the 2 bands indicate the enrichment compare to Ctls unsubtracted and Ctls-SH subtracted samples.
doi:10.1371/journal.pone.0029440.g002

expressed cDNA clones were identified based on the hybridization signal intensities observed between the four membranes. The positive clones had 1) a stronger hybridization signal with the SH-Ctls probe than with SH probe, 2) a weaker hybridization signal with the Ctls-SH probe than with the Ctls probe, and 3) a stronger hybridization signal with the SH-Ctls probe than with the Ctls-SH probe. Representative differential screening results are illustrated in Figure 3. Of the 950 cDNA clones screened, 294 were identified as strongly expressed and were analyzed on agarose gel. Sequencing performed on single band PCR amplicons generated a total of 224 clones with adequate sequencing results for BLAST analysis and Genbank deposition (accession numbers from GH613643 to GH613840).

The first group contained 167 sequences with known function, 20 of which were redundant. These sequences were further categorized based on their biological pathways (Table S2, 147 sequences). The second group contained 57 sequences previously characterized, but with unknown functions; 9 of these sequences were redundant (Table S2, 48 sequences). In group I, there were 14 genes related to regulatory proteins, 14 to immune signaling molecules, 13 to intracellular signaling component pathways, 10 to immune response, 5 to cell growth and proliferation, 5 to free radical metabolism (Table S2, 61 genes). There were 86 additional genes with known cell function (6 to transmembrane proteins, 10 to structural proteins, 4 to extracellular proteins, 1 to complement components, 4 to gene transcription, 2 to cell adhesion molecules,

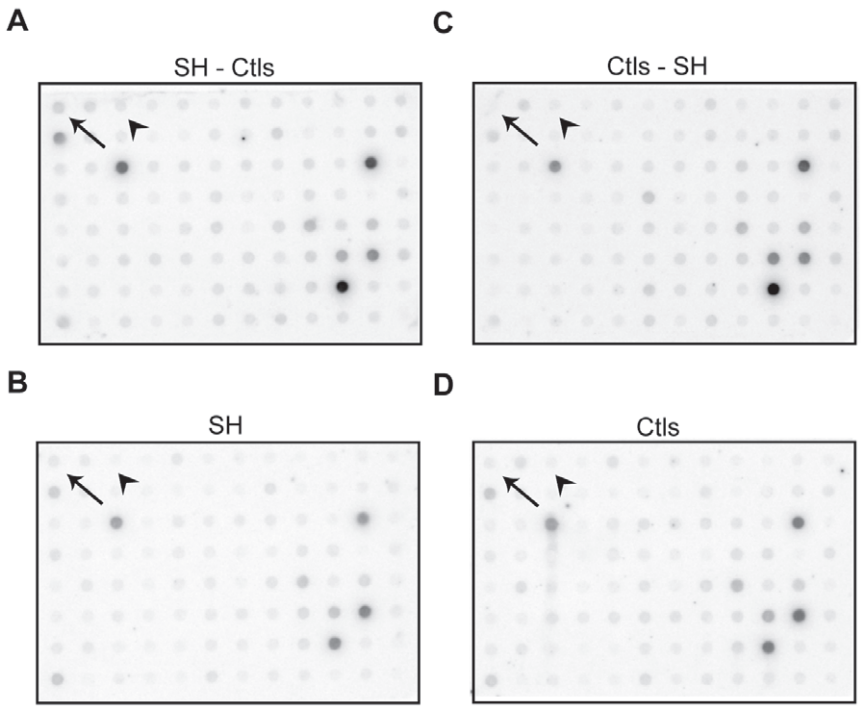


**Figure 3. Differential hybridization screening.** Representative differential screening results of macroarrays of the SH-Ctls library. Four identical membranes were dot-blotted with PCR products obtained by SSH. The membranes were then hybridized with four different probes: SH-Ctls subtracted cDNAs (A), SH unsubtracted cDNAs (B), Ctls-SH subtracted cDNAs (C) and Ctls unsubtracted cDNAs. The arrow in the top left corner indicates the positive control (*LCN2*). The arrow head indicates an example of differentially expressed genes in SH compare with Ctls.
doi:10.1371/journal.pone.0029440.g003

2 to metal ion binding, 24 to DNA/RNA associated proteins, 11 to transport proteins, 13 to metabolic enzymes, 4 to proteolytic enzymes and 5 to protein binding).

### Gene expression analysis

To confirm the differential gene expression pattern in heaves-affected horses, mRNA expression was compared by qPCR in individual heaves and control horses before and after challenge. The 22 genes selected for validation were chosen because they had previously been associated with asthma, or because of their possible contribution to airway inflammation and remodeling. They included *LCN2*, collagen type I alpha 2 (*COL1A2*), collagen type III alpha 1 (*COL3A1*), protein phosphatase 3 catalytic subunit beta (*PPP3CB*), glypican 4 (*GPC4*), versican (*VCAN*), chemokine (C-C motif) ligand 5 (*CCL5*), decorin (*DCN*), major histocompatibility complex class II invariant chain CD74 molecule (*CD74*), dedicator of cytokinesis 1 (*DOCK1*), fucosidase alpha-L-1 (*FUCA1*), mitochondrial translational release factor 1-like (*MTRF1L*), NHL repeat containing 2 (*NHLRC2*), prostaglandin D2 receptor (*PTGDR*), leukotriene A-4 hydrolase (*LTA4H*), endothelin receptor type A (*EDNRA*), chemokin binding protein 2 (*CCBP2*), insulin-like growth factor I (*IGF1*), gamma actin (*ACTG1*), vimentin (*VIM*), TRPC4 associated protein (*TRPC4AP*) and Rho GTPase activating protein 25 (*ARHGAP25*). *LCN2*, *COL1A2*, *PPP3CB*, *VCAN*, *DCN*, *CD74*, *NHLRC2*, *IGF1*, *FUCA1* and *LTA4H* mRNA were significantly increased in heaves-affected horses after the challenge compared to baseline, in contrast to the mRNA expression in controls, which remained stable during the study. Similarly, *PTGDR* and *MTRF1L* also showed a significant increase in heaves-affected horses after challenge compared to baseline, but in addition, the baseline mRNA expression was significantly higher in the control group compared to the heaves-affected group. The expression of *ARHGAP25* and *ACTG1* mRNA were significantly decreased after challenge compared to baseline in control horses only. *ARHGAP25*, *EDNRA* and *ACTG1* were also significantly different between groups at baseline, control horses having higher mRNA expression. There were no significant differences between groups and between time points in each group in *COL3A1*, *GPC4*, *CCL5*, *DOCK1*, *CCBP2*, *TRPC4AP* and *VIM*. Figure 4 represents the mRNA expression using qPCR for six of the genes found to be upregulated with SSH.

Quantification for genes of interest is expressed as absolute concentration because all reference genes tested showed significant ($p<0.05$) increase in the lung tissue of heaves-affected horses after the antigen challenge compared to baseline. The reference genes tested were *GAPDH*, ubiquitin C (*UBC*), b-glucuronidase (*GUSB*), ß2-microglobulin (*B2M*), peptidylprolyl isomerase A (*PPIA*), large ribosomal protein P0 (*RPLP0*), and ribosomal protein S9 (*RPS9*). Reference gene analysis using dedicated softwares further confirmed that the stability of these genes was highly dependent on horses' clinical status, precluding their use for normalization. In view of these results, total RNA and reverse transcribe (RT) reactions were quantified using a spectrophotometer (NanoDrop ND-1000, NanoDrop products, Wilmington, DE, USA) and used to normalize cDNA quantity in the PCR reactions [26]. Lastly, to ensure that unidentified biases were not introduced during sample analysis, the upregulation of *GAPDH*, *PPIA*, *LTA4H*, *PPP3CB* and *LCN2* in horses with heaves after antigen stimulation was confirmed (data not shown) using samples of lung tissues from the same animals but archived using a different technique of preservation (RNA later, Ambion, Austin, TX, USA), RNA extraction (TRIzol® Invitrogen, Carlsbad, CA, USA) and a different enzyme for RT reactions (AMV, Roche Diagnostics Corp, Laval, QC, CA).

## Discussion

Large-scale expression studies have highlighted complex interactions occurring between genes and environment in the central asthmatic airways [27] and allowed the discovery of novel pathways implicated in the disease inflammatory and remodeling processes. In the present study, we generated a reciprocal cDNA library representing mRNA specific to the peripheral lung of asthmatic horses during exacerbation. This led to the identification of 195 genes, 75.4% of which corresponded to genes with known sequences and functions and 24.6% to uncharacterized cDNAs. Quantitative PCR confirmed the differential gene expression pattern in 15 of the 22 genes evaluated. The functions of many of these genes were related to inflammation, remodeling, and smooth muscle biology, possibly representing new therapeutic targets for asthma.

### Genes associated with remodeling and smooth muscle contraction

Inflammation and repair of injured lung tissues in asthma results in an increased thickness of the airway wall leading to reduced baseline airway caliber and exaggerated airway narrowing, phenomena that are accentuated in allergen-induced bronchospasm [28]. Of particular importance to asthma is the increased airway smooth muscle (ASM) mass observed in human subjects (reviewed by [29]) and in equine heaves [14,15]. Not only are ASM cells increased in number or size and contribute to the bronchospasm, but they show, at least *in vitro*, some phenotype plasticity in response to allergen challenge, including de-differentiation to a more synthetic type capable of producing an extracellular matrix (ECM), various cytokines, and growth factors (reviewed by [30]). However, the molecular pathways responsible for these changes are poorly defined. Herein, we identified at least 13 genes that have been linked to smooth muscle biology. Four genes (*ARHGAP25*, phosphatidylinositol transfer protein alpha (*PITPNA*), phosphatidylinositol-specific phospholipase C X domain containing 3 (*PLCXD3*), and pyruvate dehydrogenase kinase isozyme 1 (*PDK1*)) identified in the lung tissues of heaves-affected horses in exacerbation are involved in the modulation of the RhoA pathway [31]. This pathway is necessary for the activation of factor serum response factor (*SRF*) by myocardin [32], one of its coactivators, which leads to the expression of contractile protein genes expression [33]. Interestingly, RhoA/Rho kinase is required for ASM contraction induced by endothelin-1 (*EDN1*) [34] and is upregulated by interleukin-4 (*IL-4*), a Th2 cytokine expressed in the airways of asthmatic patients [35]. Our results are thus in agreement and extend those of *in vitro* and animal studies, and further support the proposal of Rho kinase inhibitors as new targets for the treatment of airway bronchoconstriction and remodeling seen in asthma (reviewed by [36]). Conversely, it is the mitogen-activated protein kinase (*MAPK*) signaling pathway that promotes smooth muscle proliferation by modulating *SRF*-transcriptional activities via the activation *Elk-1* [32,33]. *MAPK1* identified in our SSH activates *Elk-1* [37], suggesting that both ASM proliferation and differentiation may coexist in the asthmatic lungs.

*PPP3CB* (also known as calcineurin) and *IGF1* identified by SSH share common signaling pathways also possibly contributing to smooth muscle phenotype switching and ECM remodeling in asthma [38,39,40,41,42]. The identification of *PPP3CB*, and one of its inhibitors, calcineurin homologous protein (*CHP*) [43], is of particular interest as *PPP3CB/NFAT* signaling is implicated in a wide range of biological responses relevant to asthma including lymphocyte activation, as well as neuronal and muscle development

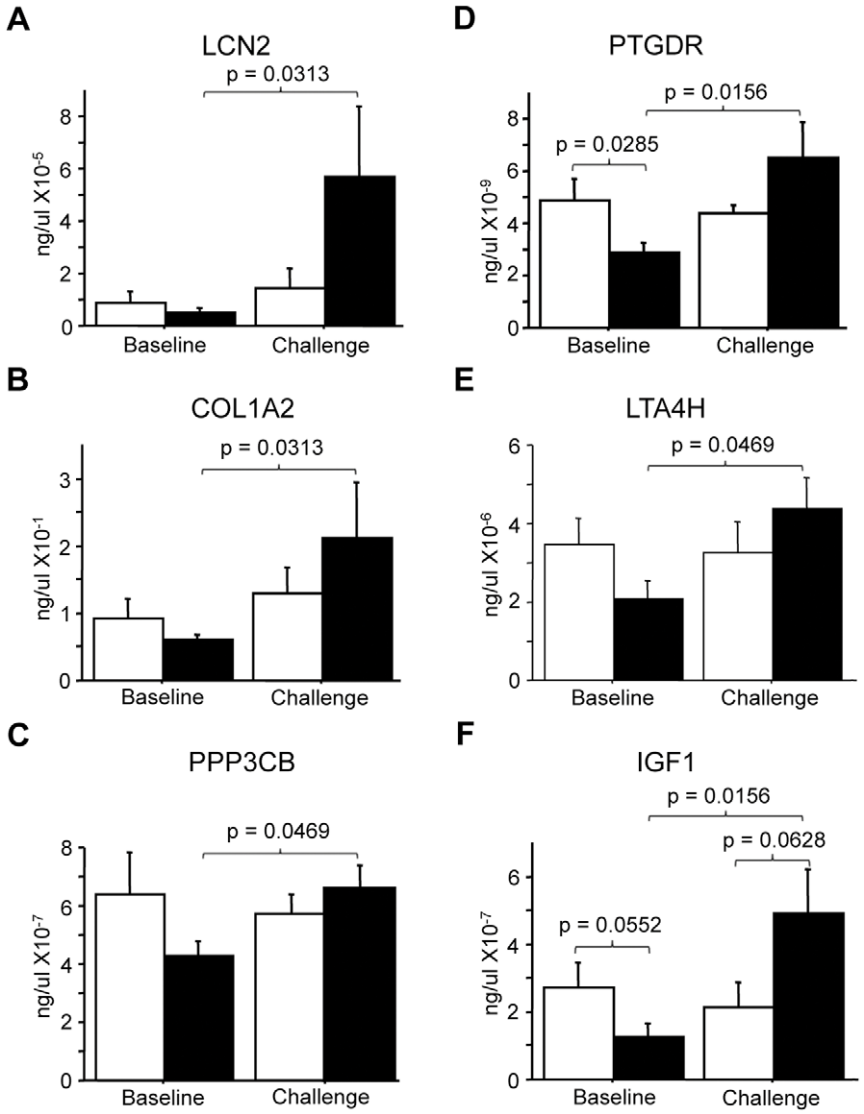


**Figure 4. Gene expression analysis.** Analysis of mRNA expression using qPCR of six genes found up-regulated with SSH. *LCN2* (A), *COL1A2* (B), *PP3CB* (C), *PTGDR* (D), *LTA4H* (E) and *IGF1* (F) were studied in six horses with heaves (black bars) and six control horses (white bars). When compared to baseline, the six genes were significantly increased in heaves-affected horses after the allergen challenge ($p<0.05$). *PTDGR* was also increased in control horses when compared to heaves-affected horses at baseline.
doi:10.1371/journal.pone.0029440.g004

[43,44,45]. While not yet investigated in lung tissues to our knowledge, *PPP3CB* activation results in muscle hypertrophy in response to increase workload in both the urinary bladder and in the heart [46,47,48]. Furthermore, alterations of the expression of the fast and slow myosin heavy chain isoforms in the obstructed bladder is *PPP3CB*-dependent [48]. Thus the *PPP3CB* pathway may participate in the increased ASM mass and the myosin heavy chain isoform switching observed in the asthmatic airways [49]. The expression of *EDN1*, a potent spasmogen for the bronchus, is increased in asthma [50], and single nucleotide polymorphisms (SNPs) have been associated with susceptibility to this disease [51]. One of its receptor, *EDNRA*, identified in our SSH, has previously been found to be upregulated in this animal model [52]. Interestingly, the *PPP3CB/NFAT* pathway discussed above has been shown to be required for at least some of the effects of *EDN1* in cardiac myocytes [39,53], and thus, are further support for their possible modulation of ASM remodeling.

*IGF1* plays a vital role in embryonic development and promotes the anabolism and the repair of various tissues in adults [54]. There are several evidences suggesting that *IGF1* may also contribute to asthma. *IGF1* is produced by human bronchial epithelial cells in response to *IL-17F* [55], a cytokine implicated in asthma. It was shown to induce the expression of alpha-smooth muscle actin and type-I collagen by human fetal lung fibroblasts [56], and to promote visceral myocyte differentiation into a contractile phenotype via the *PPP3CB/NFAT* pathway [57,58]. The increased expression of *IGF1* in the peripheral lung tissue of horses with heaves during exacerbation is thus of interest to asthma, especially in the light that *IGF1* neutralizing antibody inhibits airway obstruction and inflammation, while preventing airway wall thickening in a mouse model of asthma [59].

There is also alteration of various components of the ECM in the asthmatic airways. These changes vary depending on size of airways, and it has been shown that an increase in the degree of

subepithelial fibrosis correlates with an increase in the severity of asthma [60]. Not surprisingly, gene expression of ECM molecules including collagens (*COL1A2*, *COL3A1*), and proteoglycans (*DCN*, *GPC4*, *VCAN*) were identified by SSH. The expression of collagen, type I, and type III led us to investigate the total collagen content in the airways of these horses which revealed to be increased (unpublished data). The increased expression of the intermediate filaments *VIM* may also be relevant as it is required for epithelial to mesenchymal transition, a phenomenon where epithelial cell properties change from non-migrational to a fibroblastic and migrational-mesenchymal cell type, which has been proposed to be contributing to the increased ASM mass observed in asthma [61].

### Genes associated with inflammation

Pulmonary inflammation is a characteristic finding in asthma and anti-inflammatory drugs are central for its control. Seven genes associated with leukotriene (*LT*)B4 metabolism or prostaglandin (*PG*)D2 activity were identified as being overexpressed in the lungs of horses with heaves during exacerbation. Those included LTA4 hydrolase which metabolizes *LTA4* in *LTB4*, PGF synthase that reduces *PGD2* and *PGH2* to *PGF2*, *PTGDR* (also named *DP1*), a *PGD2* receptor involved in the regulation of Th2-type driven inflammation [62], and *CNOT7* (CCR4-NOT transcription complex), a repressor of the retinoid X beta receptor (*Rxrb*) [63], which forms a heterodimeric complex with the nuclear receptors *PPARs* (peroxisome proliferator-activated receptor). *LTB4* is an arachidonic acid metabolite synthesized by various cell types when activated by inflammatory stimuli. *LTB4* was first described as a potent chemoattractant and activator of neutrophils, the predominant airway cell population present in heaves, and in some asthmatic patients [64]. It is now recognized that *LTB4* also exerts these effects on other cell types involved in airway inflammation [65] and it has also been suggested that it is implicated in T cell trafficking and asthmatic inflammation [66]. Further support for a role of *LTB4* in asthma is its increase in exhaled breath condensate of affected patients [67], and the attenuation of allergic airway inflammation and hyperresponsiveness by *LTA4H* inhibition [68]. However, the effects of *LTA4H* are complex, as it can also limit tissue damage-induced neutrophilia through its aminopeptidase activity which degrades proline-glycine-proline (PGP), a collagen breakdown product possessing potent neutrophil chemotactic activity [69]. *PGD2* is also an arachidonic acid metabolite that is released in large quantities by mast cells during anaphylaxis. Other cell types present in lung tissues such has dendritic cells, macrophages, eosinophils, Th2 cells, and endothelial cells may also produce *PGD2*, and contribute to asthmatic inflammation [66]. *PGD2* exerts its effects by activating two distinct G protein-coupled receptors, including the *PTGDR* identified by SSH. It has been recently proposed that *PTGDR* may regulate *PGD2*-directed T-cell trafficking and Th2-dependent airway inflammation [62,66]. These results suggest that the pharmacological modulation of these lipidic mediators represent possible novel therapeutic targets for the treatment of human asthma [70].

In summary, we have identified genes and pathways relevant to the asthmatic inflammation and remodeling that were upregulated in the peripheral lung tissues of horses with heaves when antigen challenged. Genes previously associated with asthma as well as novel pathways were also identified. These genes encompass a range of biological processes with pathways related to ASM and ECM remodeling, and inflammation being notable. Our results suggest that targeting *RhoA*, *PPP3CB*, *EDN1*, and *IGF1* signaling pathways may represent appropriate targets for anti-remodeling therapies, especially for the control ASM hypertrophy, while anti-inflammatory effects may possibly be achieved by drugs modulating *LTB4* and *PGD2*.

## Supporting Information

**Table S1 Sequences of primer pairs used for PCR analysis.**
(DOCX)

**Table S2 Identification and functional classification of differentially expressed transcripts in horses with heaves during airway obstruction when compared to healthy controls and asymptomatic asthmatic horses.**
(DOCX)

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## Author Contributions

Conceived and designed the experiments: JPL JL. Performed the experiments: JLL ML ALL AC CL. Analyzed the data: JPL JLL AC CL. Contributed reagents/materials/analysis tools: JPL JL CL. Wrote the paper: JPL JLL.

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# Annexe 5

## Effect of Long-Term Fluticasone Treatment on Immune Function in Horses with Heaves

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Sommaire
Cet article décrit l'effet sur le système immunitaire d'un traitement prolongé avec des corticostéroïdes administrés par inhalation à des chevaux atteints du souffle. Bien qu'il ait été démontré chez d'autres espèces que les corticostéroïdes administrés par inhalation ont moins d'effets secondaires que les corticostéroïdes administrés de façon systémique, aucune étude n'avaient porté sur les effets d'un traitement prolongé chez les chevaux et aucune n'avait investigué les effets sur le système immunitaire de façon aussi détaillée. La conclusion de cette étude est que l'administration de fluticasone sur une période de un an à doses thérapeutiques n'a pas d'effet détectable sur l'immunité innée et acquise (humorale et à médiation cellulaire) et ne devrait pas compromettre la réponse vaccinale ni la défense contre les pathogènes des chevaux adultes.

**Contribution**
J'ai contribué à l'élaboration du protocole expérimental (10%), à l'administration des traitements et au suivi quotidien des chevaux (40%), au prélèvement des échantillons (10%), à l'analyse des données (10%) et à la rédaction de l'article (10%).

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# Effect of Long-Term Fluticasone Treatment on Immune Function in Horses with Heaves

J. Dauvillier, M.J.B. Felippe, D.P. Lunn, A. Lavoie-Lamoureux, M. Leclère, G. Beauchamp, and J.-P. Lavoie

**Background:** Corticosteroids currently are the most effective pharmacological treatment available to control heaves in horses. Systemically administered corticosteroids have been shown to alter immune response in horses, humans, and other species. Aerosolized administration theoretically minimizes systemic adverse effects, but the effect of inhaled corticosteroids on immune function has not been evaluated in horses.

**Objectives:** To evaluate the effects of prolonged administration of inhaled fluticasone on the immune system of heaves-affected horses.

**Animals:** Heaves-affected horses were treated with inhaled fluticasone (n = 5) for 11 months or received environmental modifications only (n = 5).

**Methods:** Prospective analysis. Clinical parameters and CBC, lymphocyte subpopulations and function, and circulating neutrophil gene expression were sequentially measured. Primary and anamnestic immune responses also were evaluated by measuring antigen-specific antibodies in response to vaccination with bovine viral antigen and tetanus toxoid, respectively.

**Results:** No clinical adverse effects were observed and no differences in immune function were detected between treated and untreated horses.

**Conclusions and Clinical Importance:** The treatment of heaves-affected horses with inhaled fluticasone at therapeutic dosages for 11 months has no significant detectable effect on innate and adaptive (both humoral and cell-mediated) immune parameters studied. These results suggest that prolonged administration of fluticasone would not compromise the systemic immune response to pathogens nor vaccination in adult horses.

**Key words:** Aerosolized; Horse; Inhaled corticosteroids; Recurrent airway obstruction.

Heaves or recurrent airway obstruction is a common disease of horses stabled for extended periods. Susceptible horses develop lower airway obstruction and neutrophilic airway inflammation with inhalation of dust present in hay and bedding.[1] Corticosteroids currently are the most effective pharmacological treatment available for the condition. However, systemic administration of corticosteroids to horses has been associated with several adverse effects, including adrenocortical suppression[2,3] and dysfunction,[4] laminitis,[5,6] hepatopathy,[6,7] muscle wasting,[7] altered bone metabolism,[8] and increased susceptibility to infection.[9–11] Systemic corticosteroids also have been shown to affect the equine immune system by inducing transient peripheral neutrophilia and lymphopenia,[12,13] changes in lymphocytes subpopulations and expression of activation markers,[14] as well as by decreasing the antibody response to vaccination.[15]

Inhaled corticosteroids now are commonly used for the treatment of heaves in horses.[16–20] This approach aims at achieving maximal concentrations of drug within the airways, while minimizing systemic adverse effects. In people, in whom inhaled corticosteroids are the first-line therapy for treatment of asthma and chronic obstructive pulmonary disease (COPD), prolonged administration of corticosteroids has been associated with adverse effects including reduction in bone density, cataracts, glaucoma, skin bruising,[21] and modulation of cellular immunity at high doses.[22] They also have been shown to induce a dose-dependent suppression of endogenous cortisol in horses[2,23–25] suggesting that systemic effects may occur in this species. Thus, the objective of this study was

**Abbreviations:**

| | |
|---|---|
| CD | cluster of differentiation |
| cDNA | complementary DNA |
| CFSE | carboxyl-fluorescein diacetate, succinimidyl ester |
| ConA | concanavalin A |
| COPD | chronic obstructive pulmonary disease |
| FACS | fluorescent-activated cell sorting |
| FCS | fetal calf serum |
| FITC | fluorescein isothiocyanate |
| GC | glucocorticoides |
| IgG | immunoglobulin G |
| IL-8 | interleukin-8 |
| LFA-1 | lymphocyte function-associated antigen-1 |
| LPS | lipopolysaccharide |
| MHC II | major histocompatibility complex class II |
| PWM | pokeweed mitogen |
| qRT-PCR | quantitative reverse transcriptase polymerase chain reaction |
| RPMI | Roswell Park Memorial Institute |
| TNF-α | tumor necrosis factor-alpha |

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to investigate the effects of long-term treatment of heaves-affected horses with inhaled fluticasone on innate and acquired (humoral and cell mediated) immune responses.

## Materials and Methods

### *Animals*

Ten heaves-affected mixed breed horses (6 mares and 4 geldings) aged 13–23 years (mean ± SD, 17.9 ± 2.9 years) weighing 410–535 kg (475 ± 38 kg) were used in this study. Horses were diagnosed with heaves on the basis of history, characteristic clinical presentation and worsening of clinical signs, pulmonary function, and bronchoalveolar lavage cytology after exposure to hay. All horses belonged to the research herd from the Respiratory Cellular and Molecular Biology Laboratory of the Université de Montréal, and were part of a larger study evaluating the reversibility of pulmonary remodeling. They were dewormed routinely and vaccinated annually (tetanus, West Nile, Eastern and Western equine encephalitis, equine herpes virus 1/4, influenza, and rabies vaccines). Horses were managed as a closed herd except for a few days in month 6 when they were hospitalized for thoracoscopy. Physical examination, CBC, and blood biochemistry profiles were performed before the study to exclude concomitant medical disorders. All experimental procedures were performed in accordance with the Canadian Council of Animal Care and approved by the University of Montréal Animal Care Committee.

### *Experimental Protocol*

Before initiation of the study, all horses were stabled and fed hay for 1–3 months to induce clinical exacerbation of respiratory disease. They then were assigned to 2 groups with similar severity of airway obstruction based on results of lung function tests (maximal variation in transpulmonary pressure [ΔPL], 44 ± 10 $cmH_2O$ in untreated group; 43 ± 10 $cmH_2O$ in fluticasone group). The 2 groups also were similar in terms of mean age and sex distribution. After a stabling period necessary to document the bronchoconstriction, horses in the untreated control group (n = 5, age, 17.4 ± 3.6 years; 3 mares, 2 geldings) were kept outside on pasture for the duration of the study and received no medications. They received a complete pelleted feed twice a day to maintain body condition (no hay) and had free access to grass in the summer. Horses in the fluticasone group (n = 5, age, 18.4 ± 2.2 years; 3 mares, 2 geldings) remained stabled and were fed hay for the first 5 months of the study, and then were kept on pasture with the untreated horses (under the same conditions) for the remaining 6 months of the study. They were administered inhaled fluticasone propionate[a] from a metered-dose inhaler via a commercially available mask,[b] at a starting dose of 2,000 μg twice a day. The dose then was adjusted as needed to keep the horses asymptomatic (from 2,000 μg q24h to 3,000 μg q12h), but, from the 6th month of study until the end, all horses in the fluticasone group received inhaled fluticasone at 2,000 μg once a day, between 7:00 and 9:00 AM. While stabled, horses were turned out in a paddock 2–4 hours each day. The study was initiated in the springtime and finished in winter of the following year.

The timeline of the study is outlined in Figure 1. Horses were observed daily during the whole study. Blood samples for CBC were collected in ethylenediaminetetraacetic acid tubes, cell counts were performed with an automated analysis system,[c] and blood smears were reviewed by a clinical pathologist. Heparinized blood samples were processed within an hour for gene expression analysis and kept on ice overnight for lymphocyte phenotyping and proliferation assays. Vaccination was done with a tetanus toxoid[d] and the infectious

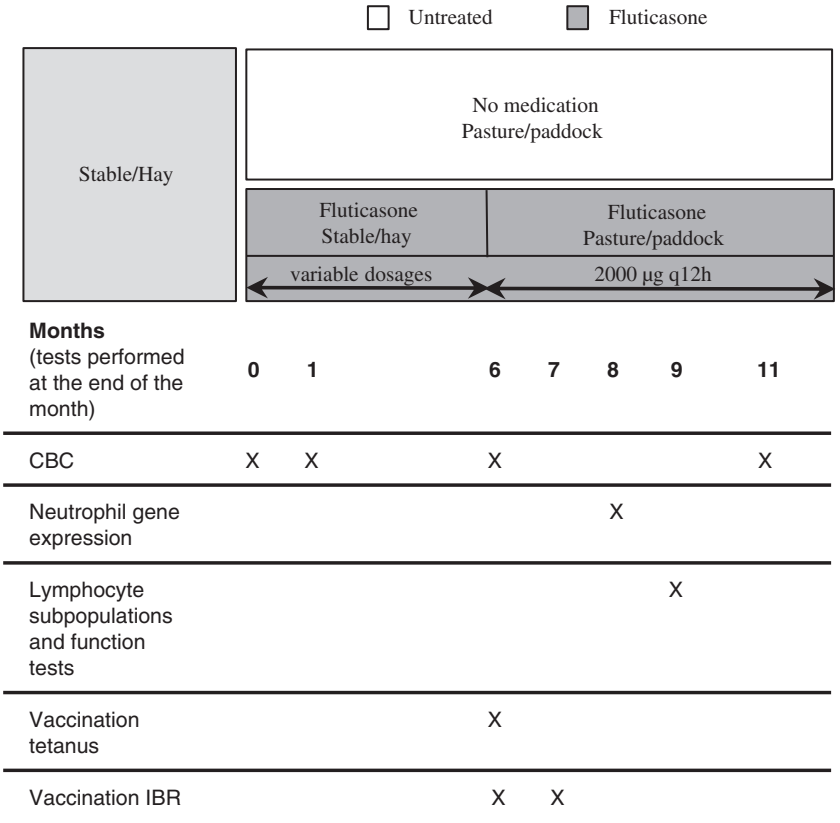


**Fig 1.** Study time line.

bovine rhinotracheitis (IBR) vaccine, which also contained bovine viral diarrhea (type I and II), parainfluenza-3 and syncytial bovine virus antigens, combined with an appropriate adjuvant.[e] The experimental vaccination protocol was initiated 2 months after the horses received their last annual vaccine booster injection. Blood samples were collected before the 1st vaccine injection and then sequentially for 4 months. Serum was separated, aliquoted, and stored at −80°C until analysis. All blood samplings were performed by jugular venipuncture in the morning (before fluticazone administration in the treated group). As part of the larger study evaluating the reversibility of pulmonary remodeling, lung biopsies were performed under thoracoscopic guidance 2 weeks before the beginning of the study, at approximately 6 months, and after the last CBC sampling at the completion of this study. Blood samples were collected either before or at least 1 month after the surgeries to prevent interference with the results.

## Lymphocyte Phenotyping, Activation State, and Proliferation Assays

Peripheral blood mononuclear cells were isolated with Ficoll gradient centrifugation as described previously.[26] Isolated cells were analyzed by flow cytometry (fluorescent activated cell sorting [FACS]) for lymphocyte antigen markers (cluster of differentiation [CD]4 clone HB61A, CD8 clone HT14A, B cell clone cz2.1).[27,28] Cell expression of major histocompatibility complex (MHC) class II molecules (clone cz11) and lymphocyte function-associated antigen-1 (LFA-1) (orCD11a/CD18, clone cz3.2) also were measured as markers of lymphocyte activation. The secondary stage used was fluorescein isothiocyanate-conjugated F(ab') fragment goat anti-mouse immunoglobulin G (IgG) (heavy + light chains) antibody. Samples were analyzed on a FACScalibur flow cytometer equipped with a 488 μm argon laser by Cell Quest Analysis software. Leukocyte subpopulations were displayed in a dot plot and gated according to size based on forward light scatter, and according to granularity based on 90° side light scatter.[29] A region was placed around lymphocytes, and data were collected on 10,000 gated cells. Results indicate percent positive cells and mean fluorescence intensity in the lymphocyte-gated area.

For proliferation assays, lymphocytes were isolated by a combined carbonyl iron and Ficoll method, and labeled with carboxyl-fluorescein diacetate, succinimidyl ester (CFSE) as described before.[26] Briefly, cells were resuspended in Roswell Park Memorial Institute (RPMI) cell culture medium with 10% fetal calf serum (FCS), antibiotics/antimycotics and mercaptoethanol solution. In another set of experiments, medium was prepared with 10% autologous serum instead of FCS. Cell suspensions were incubated with or without addition of pokeweed mitogen (PWM) or concanavalin A (ConA). Cells were harvested at 96 hours and tested for CFSE fluorescence with flow cytometry (FL1). A decrease in CFSE mean fluorescence was considered proportional to the division of fluorescence dye to daughter cells at each cell division. Results were analyzed using the percentage of cells with lower fluorescence than the control nonstimulated cells.

## Gene Expression by Peripheral Blood Neutrophils

***Neutrophil Isolation and Culture Conditions.*** Peripheral blood neutrophils were isolated using immunomagnetic selection (magnetic-activated cell sorting) as reported previously.[30] Briefly, neutrophils were retrieved from the leukocyte-rich supernatant by sequential incubation with primary monoclonal antibody[f] and secondary rat anti-mouse IgM antibody conjugated to paramagnetic microbeads[g] before being loaded on a ferromagnetic LS separation column.[g] Cytospin slides were prepared[h] and stained with Protocol Hema 3[i] for differential counting of >400 cells to assess neutrophil purity. Viability was determined by trypan blue exclusion.

Purified neutrophils were suspended at $5 \times 10^6$ cells/mL in culture medium RPMI 1640[j] supplemented with 10% heat inactivated low-endotoxin FCS,[j] 4 mM L-glutamine,[j] 100 U/mL penicillin, and 100 μg/mL streptomycin.[j] The cells were incubated in 6-well suspension plates for 5 hours at 37°C, 5% $CO_2$ in the presence of 100 ng/mL lipopolysaccharide (LPS) from *Escherichia coli* 0111:B[k] and 10 nM fMLP[k] to induce proinflammatory cytokine gene expression.[30] Unstimulated neutrophils were used as control (resting). At the end of incubation time, $10^6$ cells per test were homogenized in TRIzol Reagent[l] and stored at −80°C until RNA extraction. Complementary DNA (cDNA) samples derived from LPS-stimulated neutrophils with or without dexamethasone ($10^{-6}$ M)[31] were used to assess the expression of glucocorticoid (GC)-responsive genes (GC receptor and glutamine synthetase).

***Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR).*** RNA extraction, reverse-transcription (500 ng total RNA), and real-time PCR were performed as described previously[32] with the Rotor-Gene Real-Time Centrifugal DNA Amplification System 3000.[m] Primers pairs were as follows (5′→3′): interleukin (IL)-8.S (sens) CTTTCTG CAGCTCTGTGTGAAG and IL-8.AS (anti-sens) GCAGACCT-CAGCTCCGTTGAC; tumor necrosis factor-alpha (TNF-α).S CTTGTGCCTCAGCCTCTTCTCCTTC and TNF-α.AS TCTT GATGGCAGAGAGGAGGTTGAC; GC receptors (GCR).S TCATTAAGCTCCCCTGGCAGAGAA and GCR.AS ATT-GAGAGTGAAACGGCCTTGGAC; glutamine synthetase (glut. Synt.).S ACTGGATTCCACGAAACCTCCAAC and glut. synt.AS GCTGCAAGTCTAGTCCGCTTAGTT; Ubiquitin.S TAGCAGTTTCTTCGTGTCCGT and Ubiquitin.AS TGTAA TCGGAAAGAGTGCGG. GCR primers were designed to amplify the precursor (unspliced) transcript encoding both the α and β GCR isoforms, which are both down-regulated by GC treatment.[33] All primers spanned exon-intron boundaries in order to prevent amplification of genomic DNA. Values were normalized using ubiquitin expression as reference gene.

## Dosage of Serum-Specific IgG

Serum titers of tetanus toxoid antigen-specific IgGb were determined by use of ELISAs as described previously.[34] Serum titers of IBR antigen-specific IgG were determined with a commercial ELISA test kit designed for the detection of IBR antibodies in bovine serum,[n] in which a secondary anti-equine monoclonal antibody (CVS39[28]) was substituted to the anti-bovine 1 provided in the kit.

## Statistical Analysis

By a repeated measures analysis of variance (ANOVA) and by a sequential Bonferroni's procedure to adjust comparison-wise α levels, peripheral blood leukocytes, lymphocytes, neutrophils, eosinophils, basophils, and monocytes were compared at each time points between treated and nontreated horses, and within each group values at each time point were compared with values at baseline. A repeated measures ANOVA was used to analyze log 10 transformed anti-tetanus and anti-IBR IgGb titers. Analysis was done by SAS v. 9.2.[o]

Unpaired Student's *t* tests were used to compare proportions of CD4+ and CD8+ peripheral blood T cells, CD4+/CD8+ ratio, expression of LFA-1 and CMH II on lymphocytes and monocytes, and proliferation of lymphocytes after stimulation between the 2 groups at 9 months. Analysis was done by Statview v. 5.0.[o]

For gene expression by peripheral blood neutrophils, data were log 10 transformed to normalize distribution. One-way ANOVA and Bonferroni's multiple comparison posthoc tests were used to

assess the in vitro effect of dexamethasone on GC-sensitive genes in cDNA samples from stimulated neutrophils. Where gene expression by neutrophils isolated from fluticasone-treated and untreated horses was compared, 2-way repeated measures ANOVA followed by Bonferroni's posthoc comparisons was used to evaluate the effect of fluticasone treatment and cell stimulation. Analysis was done by Graphpad Prism 5 software.[p]

## Results

### *Horses*

Inhaled fluticasone administration was well tolerated by all horses, and administration took approximately 4 minutes. Airway obstruction present at baseline was resolved for more than 2 months when immune function parameters were studied. During the 11 months of the study, 3 horses developed clinical disorders, 2 in the fluticasone group and 1 in the untreated group. Approximately 6 months after the beginning of the study, a fluticasone-treated gelding had a suspected episode of postthoracoscopy telogen effluvium diagnosed on the basis of clinical findings and histological examination of skin biopsies. Another treated horse developed a corneal ulcer believed to be traumatic, which resolved with topical antibiotic treatment. Finally, a horse in the untreated group developed a warm and painful swelling of the left front leg, associated with a moderate peripheral blood neutrophilia ($12.5 \times 10^9$/L; reference range, 5.5–$12.5 \times 10^9$/L) and hyperfibrinogenemia (5 g/L; reference range, 1–4 g/L) 5 months after the beginning of the study. The problem resolved with a 5-day course of penicillin and phenylbutazone. The latter episode began 2 weeks before the 6-month CBC and vaccination.

### *Differential White Blood Cell Count*

All values remained within reference ranges at all time points (Fig 2). The only significant change observed was a decrease in monocyte count in untreated horses between baseline and 1 month ($P = .0045$; Fig 2D).

### *Peripheral Blood Lymphocyte Phenotyping, Activation State, and Proliferation Assays*

No significant differences between fluticasone and untreated groups were observed in the proportions of CD4+ T cells ($P = .45$), CD8+ T cells ($P = .89$), and B cells ($P = .83$), and in the CD4+/CD8+ T-cell ratio ($P = .68$; Fig 3A–D). There was no significant difference in the proportion of lymphocytes expressing MHC class II molecule (90.5 ± 3.1 and 86.3 ± 6.4% in the fluticasone and untreated group, respectively, $P = .22$) and LFA-1 molecule (96.4 ± 0.7 and 95.1 ± 2.6%, $P = .30$). The mean fluorescence intensity also was comparable between the 2 groups for MHC class II (Fig 3E) and LFA-1 (Fig 3F).

The lymphocyte proliferation assays revealed no significant difference between fluticasone-treated and untreated horses with either PWM or Con A stimulation after 4 days of incubation in the presence of either FCS (Fig 3G and 3H) or autologous serum (results not shown).

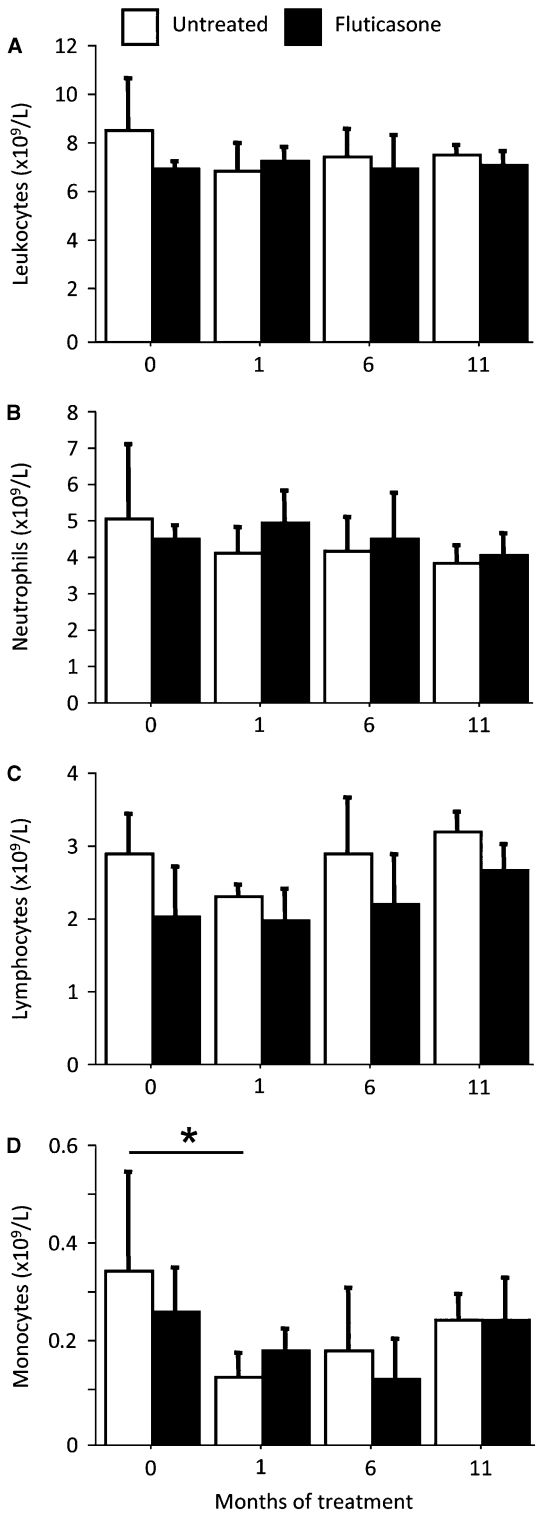


**Fig 2.** Peripheral blood leukocyte (**A**), segmented neutrophil (**B**), lymphocyte (**C**), and monocyte (**D**) counts from fluticasone-treated (black bars) and untreated (white bars) heaves-affected horses before and after 1, 6, and 11 months of treatment. Mean ± SD. $^*P < .05$.

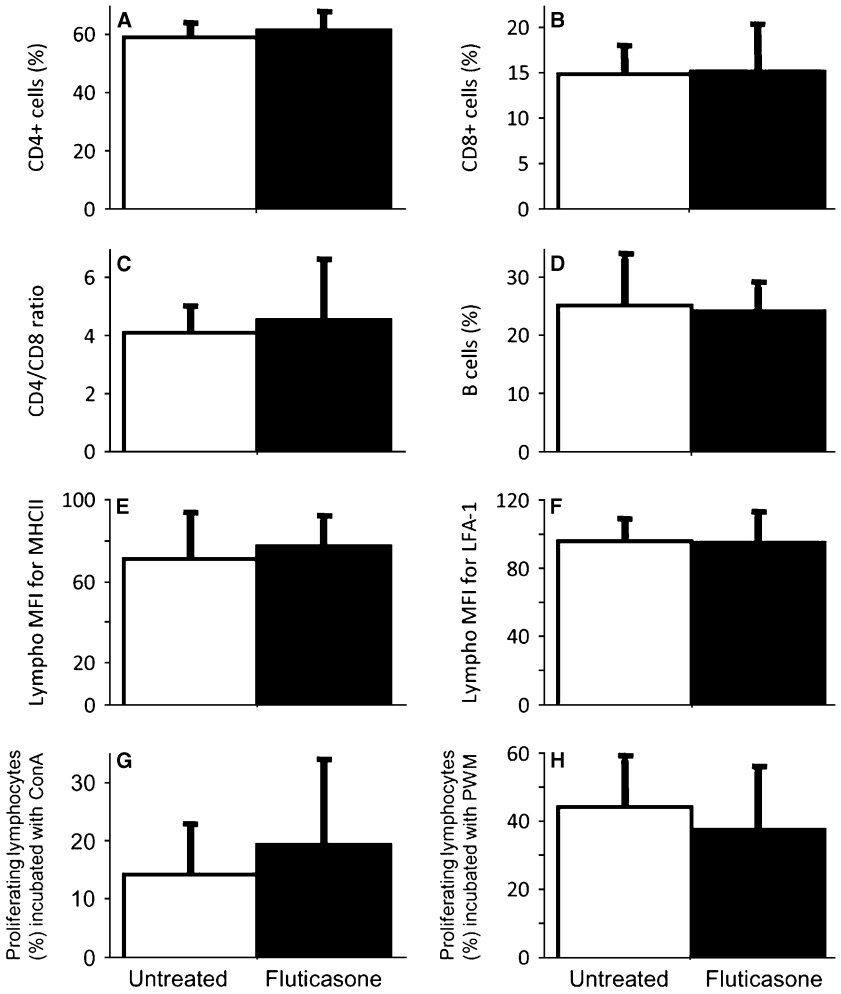


**Fig 3.** Peripheral blood CD4+ T lymphocyte (**A**), CD8+ T lymphocyte (**B**), CD4/CD8 T lymphocyte ratio (**C**), and B lymphocyte distributions (**D**). Expression (mean fluorescence intensity) of major histocompatibility complex (MHC) class II (**E**) and lymphocyte function-associated antigen-1 (LFA-1) (CD11a/CD18) (**F**) molecules on peripheral blood lymphocytes. Percentage of proliferating lymphocytes (lower mean fluorescence than control nonstimluated cells) after 4 days of incubation in vitro with concanavalin A (ConA) (**G**) or pokeweed mitogen (PWM) (**H**) and fetal calf serum in the medium; similar results were obtained with autologous serum in the medium. All tests were done in heaves-affected horses after 9 months of inhaled fluticasone treatment (black bars) or no treatment (white bars). Mean ± SD.

### *Peripheral Blood Neutrophil Gene Expression*

The purity and viability of isolated neutrophils was 98.96 ± 0.74 and 98.44 ± 1.15% (mean ± SD), respectively. An 8-month treatment period with fluticasone did not cause differences in IL-8 and TNFα mRNA expression in resting and stimulated neutrophils (Fig 4A), nor did it decrease GC receptor mRNA expression or up-regulate glutamine synthetase in resting and stimulated neutrophils when compared with untreated horses (Fig 4B). Conversely, significantly increased glutamine synthetase ($P < .05$) and decreased GCRs expression ($P < .01$) was quantified in cDNA samples from in vitro dexamethasone-treated neutrophils (not shown, n = 3), performed as a positive control.

### *Serum Anti-Tetanus IgGb Titers*

Detectable serum anti-tetanus toxoid IgGb titers were present in all horses before vaccination (Fig 5). Booster vaccination at 6 months (Time 0 on Fig 5) resulted in development of an antigen-specific IgGb response ($P < .0001$) similar in both group of horses ($P = .66$).

### *Serum Anti-IBR IgG Titers*

As expected, all horses had negative IBR titers before vaccination. Vaccination with a bovine multivalent vaccine including IBR antigens at 6 and 7 months resulted in development of anti-IBR IgG ($P < .0001$) of a similar magnitude ($P = .77$) in both groups of horses (Fig 6).

## Discussion

Prolonged administration of corticosteroids may be required to control airway obstruction in heaves-affected horses when appropriate environmental dust control is not implemented. Because of the adverse effects that have been observed with the oral or injectable use of corticosteroids, treatment duration is usually short, from

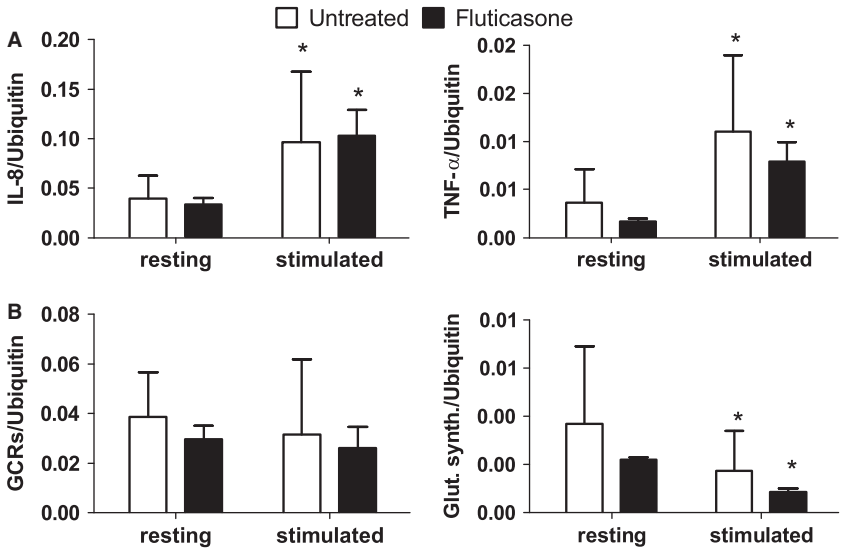


**Fig 4.** Peripheral blood neutrophil gene expression using quantitative reverse transcriptase polymerase chain reaction. Relative gene expression of proinflammatory interleukin (IL)-8 chemokine and tumor necrosis factor-alpha (TNF-α) cytokine (**A**) and glucocorticoid-responsive genes (glucocorticoid receptor [GCRs]; gluthamine synthetase) (**B**) by resting and stimulated (lipopolysaccharide 100 ng/mL and fMLP 10–8 M) neutrophils isolated from fluticasone-treated and untreated heaves-affected horses. Gene expression was normalized to ubiquitin expression (reference gene). Mean ± SD. $^{*}P < .05$: significant difference between resting and stimulated neutrophils within the same group.

days to weeks. Although better tolerated than when systemically administered in human patients, inhaled corticosteroids have nevertheless been associated with adverse effects, including a decrease in the immune response[22] and increased susceptibility to infection when administered over extended periods.[35] The risk of infection was increased especially in elderly patients and in those with severe airway obstruction, 2 key features of heaves in horses. In the present study, no adverse alterations of the immune system or clinical adverse effects were observed over an 11-month period of administration of fluticasone propionate in horses with heaves.

Innate immunity is responsible for the initial response to infectious agents, and it was evaluated here by measuring peripheral blood leukocyte count and neutrophil transcriptional response to bacterial products ex vivo (refer to Fig 1 for measurement timeline). No significant alterations in these parameters were observed with fluticasone treatment. Neutrophil and monocyte counts remained within reference ranges at all time points and did not significantly vary over time in the fluticasone group. These results are in contrast with the transient peripheral blood neutrophilia observed after a single systemic administration of corticosteroids.[12–14,36] However, and in agreement with our findings, inhaled beclomethasone did not alter differential white cell count over a 22-month treatment course in asthmatic children.[37] The decrease in peripheral monocyte count in the untreated group at 1 month was unexplained.

Because factors other than cell number can affect innate immune function, we also investigated possible down regulation of proinflammatory cytokine

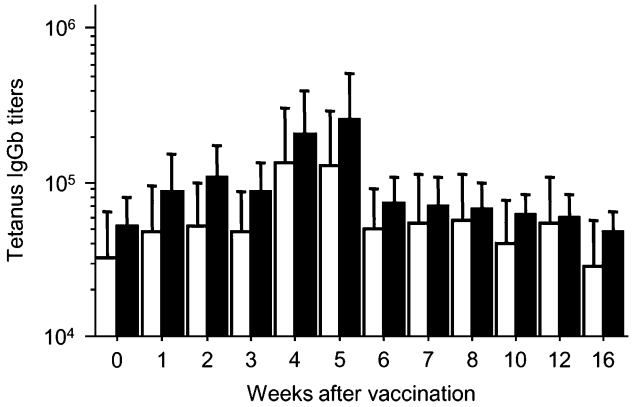


**Fig 5.** Tetanus-specific IgGb titers after vaccination with tetanus toxoid (week 0) in fluticasonetreated (dark bars) and untreated (clear bars) heaves-affected horses. Response to vaccination was significantly different at 2 and 4 weeks ($P < .05$) within groups, and similar between groups ($P > .05$). Mean ± SD.

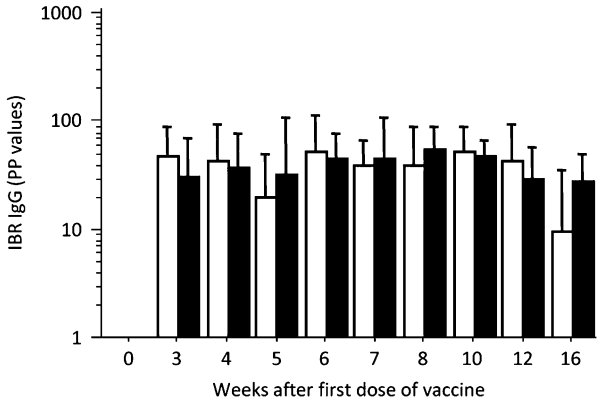


**Fig 6.** Infectious bovine rhinotracheitis (IBR)-specific IgG titers after vaccination with inactivated IBR at weeks 0 and 3 in fluticasone-treated (dark bars) and untreated (clear bars) heaves-affected horses. Response to vaccination was significantly different ($P < .05$) within groups at 3 weeks, and similar between groups ($P > .05$). Mean ± SD.

production by neutrophils after 8 months of treatment. We found no differences in IL-8 and TNFα mRNA baseline expression, and observed an appropriate response with LPS and fMLP stimulation in vitro. These results contrast with the inhibition of equine neutrophil respiratory burst and LPS-induced TNFα and IL-8 gene transcription by dexamethasone observed in vitro.[31] Accordingly, neither of the GC-responsive genes assessed (GC receptors and glutamine synthetase) were altered in peripheral blood neutrophils from fluticasone-treated horses in this study. These results support minimal if any exposure of circulating neutrophils to fluticasone and any active metabolite.

To evaluate a possible alteration of the acquired immune system, we first studied the lymphocyte count at 0, 1, 6, and 11 months. In contrast with the transient lymphopenia observed in horses after a single systemic administration of corticosteroids,[12–14,36] no significant changes in lymphocyte counts were observed with fluticasone in the present study. Similar to our findings, no change in lymphocyte counts was observed in beclomethasone-treated children.[37] We further evaluated the peripheral blood lymphocyte subpopulation distribution (CD4+ and CD8+ T cells, and B cells) and the expression of cell surface molecules (MHC class II and LFA-1) after 9 months of fluticasone inhalation, and found no changes in those parameters. Equine lymphocytes constitutively express the MHC class II and the integrin LFA-1 molecules. The function of MHC class II in lymphocytes is unknown, but expression levels have been associated with lymphocyte maturation.[38] The expression of integrins is up-regulated when cells are activated, and they facilitate cell-to-cell interaction for costimulation. Our results contrast with those reported in horses and human subjects after systemic administration of corticosteroids. In horses, for example, a decrease in total lymphocyte count and CD4+/CD8+ ratio and an increase in the expression of LFA-1 in leukocytes was observed for 48 hours after administration of a single 0.025 mg/kg IV dose of dexamethasone.[36] Similarly, in human subjects, prednisolone and dexamethasone (PO or IV) cause a decrease in total lymphocyte, T (CD4+ and CD8+), and B cell counts, with the CD4+ T cell distribution being more severely affected.[39,40] Studies on the effects of administration of inhaled corticosteroids on cell-mediated immunity in human and animal subjects led to conflicting results. A 22-month period inhaled beclomethasone in asthmatic children did not alter lymphocyte subpopulation distributions[37] nor did several weeks of treatment with fluticasone in healthy dogs,[41] or flunisolide in healthy and asthmatic cats.[42] Contrary to the effects observed after administration of a single dose[43,44] and our results, inhaled fluticasone administered for 4 weeks to healthy volunteers caused a decrease in activated CD4+ and CD8+ T cells.[22] Duration of treatment, relatively higher dosage in the study in humans, and health status of the subjects could explain these differences. We further measured the proliferative capacity of lymphocyte in response to mitogens. Proliferation was similar in treated and untreated horses at 9 months, whether lymphocytes were exposed to ConA (T-cell specific) or to PWM (B and T cells). This response was independent of the presence of autologous horse serum, which could have promoted a more sustainable effect comparable to the in vivo condition. These results are in agreement with studies in human and animal subjects after inhaled or systemic corticosteroid administration.[13,14,22,37] Only prednisolone PO has been shown to induce a transient (<24 hours) decrease in phytohemaglutinin-induced lymphocyte proliferation.[39]

Humoral immunity was investigated by measuring the primary response to an unknown antigen to horses (IBR antigen) and the anamnestic response to tetanus toxoid. Both groups responded with a similar increase in titers against these 2 antigens, despite 6 months of treatment with fluticasone. This finding contrasts with the almost complete abrogation of IgGa and IgGb response to a bovine viral vaccine observed with dexamethasone (0.2 mg/kg IM, twice a week) in horses.[15] However, these results are in agreement with the normal IgG vaccinal response of COPD and asthma patients when treated with inhaled corticosteroids,[45–47] and after the administration of a single dose of dexamethasone to healthy horses.[36]

To the authors' knowledge, no direct effect of the horse's environment on its systemic immune system has been established. However, from the 6th month of the study, all horses were out in paddocks or pastures. Thus, neutrophil gene expression measurement and lymphocyte function tests were performed when both groups of horses had been out in pasture for 2 and 5 months, respectively. These time points were chosen in order to prevent a possible effect of variable environments on the parameters studied.

In summary, this study shows that long-term treatment of heaves-affected horses with inhaled fluticasone at the therapeutic dosage has no detectable effect on the innate and acquired humoral and cell-mediated-immune parameters studied. These results indicate that this treatment would not preclude the use of vaccines in heaves-affected horses.

## Footnotes

[a] Flovent, Glaxo Wellcome, Mississauga, ON, Canada
[b] Equine Aeromask, Trudell Medical International, London, ON, Canada
[c] Advia 120 Hematology System, Siemens Healthcare Diagnostics, Deerfield, IL
[d] Tetanus Toxoid, Serial No.: 1630104B, Fort Dodge Wyeth Animal Health, IA
[e] Triangle 4 + Type II BVD, Serial No.: 178191A, Fort Dodge Wyeth Animal Health
[f] #DH24A, VMRD Inc, Pullman, WA
[g] MACS, Miltenyi Biotec, Auburn, CA
[h] Cytospin 2, Shandon, Southern Instruments, Sewickley, PA
[i] Fisher Scientific, Ottawa, ON, Canada
[j] GIBCO, Invitrogen, Burlington, ON, Canada
[k] Sigma-Aldrich, Oakville, ON, Canada
[l] Invitrogen
[m] Corbett Research, Montreal Biotech, Montreal, QC, Canada
[n] Infectious Bovine Rhinotracheitis (IBR-Ab) Svanovir, Savnova Biotech Ab, Uppsala, Sweden
[o] SAS Institute Inc, Cary, NC
[p] GraphPad Software Inc, La Jolla, CA

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# Annexe 6

Article 1 en version PDF.

# Effect of Antigenic Exposure on Airway Smooth Muscle Remodeling in an Equine Model of Chronic Asthma

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**Recent studies suggest that airway smooth muscle remodeling is an early event in asthma, but whether it remains a dynamic process late in the course of the disease is unknown. Moreover, little is known about the effects of an antigenic exposure on chronically established smooth muscle remodeling. We measured the effects of antigenic exposure on airway smooth muscle in the central and peripheral airways of horses with heaves, a naturally occurring airway disease that shares similarities with chronic asthma. Heaves-affected horses ($n = 6$) and age-matched control horses ($n = 5$) were kept on pasture before being exposed to indoor antigens for 30 days to induce airway inflammation and bronchoconstriction. Peripheral lung and endobronchial biopsies were collected before and after antigenic exposure by thoracoscopy and bronchoscopy, respectively. Immunohistochemistry and enzymatic labeling were used for morphometric analyses of airway smooth muscle mass and proliferative and apoptotic myocytes. In the peripheral airways, heaves-affected horses had twice as much smooth muscle as control horses. Remodeling was associated with smooth muscle hyperplasia and *in situ* proliferation, without reduced apoptosis. Further antigenic exposure had no effect on the morphometric data. In central airways, proliferating myocytes were increased compared with control horses only after antigenic exposure. Peripheral airway smooth muscle mass is stable in chronically affected animals subjected to antigenic exposure. This increased mass is maintained in a dynamic equilibrium by an elevated cellular turnover, suggesting that targeting smooth muscle proliferation could be effective at decreasing chronic remodeling.**

**Keywords:** asthma; animal model; peripheral airways; heaves

### CLINICAL RELEVANCE

Recent studies suggest that airway smooth muscle remodeling is an early event in asthma, but whether it remains a dynamic process late in the course of the disease, and how antigenic exposure affects established remodeling, are unknown. We showed that a 30-day antigenic exposure had little effect on established remodeling in diseased animals, despite the development of inflammation and bronchoconstriction. In peripheral airways, airway smooth muscle remodeling appears to be maintained in a dynamic equilibrium by an elevated turnover with *in situ* proliferation, suggesting that targeting airway smooth muscle proliferation may be effective at decreasing its mass.

Increased airway smooth muscle (ASM) mass is a prominent feature of patients with asthma, and may play a central role in allergen-induced bronchospasms and airway hyperresponsiveness to nonspecific agonists (1). ASM remodeling was demonstrated in the airways of patients with asthma, whether samples were obtained from lung resections or autopsy specimens (2–5), or from endobronchial biopsies (6–9). Although ASM remodeling is considered a target for novel therapies (10, 11), the processes leading to and, to a greater extent, maintaining ASM thickening in chronic disease are unknown. The question is not trivial, because therapeutic approaches targeting established remodeling would differ if the remodeling was the result of ongoing proliferation or else a decrease in cellular death. This is of particular interest in small airways, where direct therapeutic interventions such as bronchial thermoplasty are not possible (12). Recent studies also highlighted the important contributions of the small airways in asthma, while recognizing the difficulty of sampling and imaging those airways (13, 14). Among the advantages of using large animal models is the possibility of repeated sampling of peripheral airways, along with the possibility of controlling their environment and treatment, but without the genetic homogeneity of inbred colonies.

Heaves is a naturally occurring disease of horses, associated with domestication and hay feeding, that shares similarities with asthma, including reversible antigen-induced bronchoconstriction, the accumulation of mucus, and airway inflammation. Ten to fifteen percent of adult horses are affected by this condition, characterized by episodes of coughing, wheezing, and exercise intolerance that can be controlled by environmental management (i.e., avoiding the offending indoor antigens, usually organic dust from poorly conserved hay), or with corticosteroids and bronchodilators (15). Similar to certain categories of asthma, intraluminal inflammation is predominantly neutrophilic, although it was linked to Th2 cytokines in some studies (16, 17). The features of airway remodeling resemble those of asthma, and include epithelial detachment and regeneration, goblet-cell hyperplasia, and increased bronchial and bronchiolar smooth muscle (18, 19). Although heaves is caused by antigens in moldy hay, it does not resemble extrinsic allergic alveolitis, because interstitial fibrosis, alveolitis, lymphocytic bronchoalveolar lavage inflammation, and restrictive pulmonary dysfunction are not characteristic of the disease (20, 21). As in humans, the reversibility of established ASM remodeling in adult horses has yet to be demonstrated.

In this study, we hypothesized that (*1*) heaves-affected horses have greater ASM mass than age-matched control horses kept in the same environment; (*2*) a month-long antigenic exposure would further increase ASM mass in heaves-affected horses; (*3*) this greater ASM mass would be at least partly attributable to hyperplasia; and (*4*) similar changes would be

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

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evident in the peripheral airways (full-thickness biopsies) and in endobronchial biopsies (partial sampling of airway walls). To test these hypotheses, we exposed heaves-affected horses and control horses to poorly cured hay, and examined ASM in peripheral and endobronchial biopsies, harvested before and after exposure, via morphometric analyses of ASM mass and markers of proliferation and apoptosis.

## MATERIALS AND METHODS

### Experimental Design

Data were collected after horses had been on pasture for more than 3 months (baseline) and after 1 day (pulmonary function and bronchoalveolar lavage only) and 30 days of stabling and exposure to poorly cured hay (antigenic exposure).

### Animals

Six heaves-affected horses and five age-matched control horses were studied. Heaves-affected horses had a well-documented 3–10-year history of reversible airway obstruction and inflammation upon exposure to hay. Horses were deemed otherwise healthy, based on physical examination, blood count, and biochemistry. Animal manipulations were performed in accordance with the guidelines of the Canadian Council for Animal Care.

### Pulmonary Function

In unsedated animals, pulmonary resistance and elastance were calculated from the flow rates obtained from a heated pneumotachograph attached to a mask, and transpulmonary pressure was derived from an esophageal catheter (22).

### Bronchoalveolar Lavage

Two 250-ml boluses of isotonic saline were instilled in the main bronchi through a 2.5-m bronchoscope (Olympus Medical Systems Corp., Tokyo, Japan), as previously described (16). Cytospins were stained with Wright-Giemsa and Toluidine blue. Additional information is available in the online supplement.

### Endobronchial Biopsies

Biopsies were performed in the contralateral lung after bronchoalveolar lavage (BAL), using disposable forceps (Olympus Medical Systems Corp.). Biopsies (median, $n = 5$; range, $n = 3$–$8$) were taken from different branching sites, starting approximately 30 cm distal to the carina and moving cranially.

### Lung Biopsies via Thoracoscopy

Peripheral lung tissue (8–12 $cm^3$) was harvested in the caudo-dorsal region of the lung from standing, sedated animals (23). Samples were fixed for 24 hours in 4% formaldehyde and embedded in paraffin.

### Immunostaining and Enzymatic Labeling

Immunohistochemical staining was performed for the colocalization of proliferating cell nuclear antigen (PCNA) with smooth muscle–specific α-actin (18). Apoptosis was detected using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay. Additional information is available in the online supplement.

### Morphometric Analysis

In peripheral lung biopsies, the ASM area of airways in cross section was measured using Image-Pro Plus software (MediaCybernetics, Carlsbad, CA). The ASM area (median number of airways per animal, 10; range, 5–17), ASM nuclei (median number per animal, 8; range, 4–17), $PCNA^+$ myocytes (median number per animal, 8; range, 1–17), and $TUNEL^+$ myocytes (median number per animal, 7; range, 4–7) were corrected by the internal perimeter squared to account for variations in airway size (24). In endobronchial biopsies, the ASM area was measured as a ratio of the biopsy area (5; 3–8), and ASM cells (median number for $PCNA^+$, 4; range, 1–5; median number for $TUNEL^+$, 3; range, 1–4) were counted in random fields over $1–2 \times 10^4$ $\mu m^2$. Measurements were performed by one investigator (M.L.) blinded to group and the time point of sample collection.

### Statistical Analysis

Group characteristics (age, weight, and gender) were analyzed according to the Mann-Whitney test, and physiologic data (function and BAL) were analyzed according to repeated-measures ANOVA with *a priori* contrasts. Morphometric data were analyzed according to paired and unpaired two-tailed $t$ tests, using an average value for each animal at each time point. Normality was assessed according to the Kolmogorov-Smirnov test and visual inspection of the data. BAL cell counts and mast-cell percentages were transformed (log and arcsine square root, respectively) before analyses. The software SAS version 9.1 (SAS Institute, Cary, NC) was used, and $P < 0.05$ was considered significant.

## RESULTS

### Animals

Ages, weights, and proportions of mares in heaves-affected horses were not statistically different from those of control horses (ages for heaves-affected horses: median, 16 years; range, 15–20 years; ages for control horses: median, 14 years; range, 11–17 years; weights of heaves-affected horses: median, 467 kg; range, 444–515 kg; weights of control horses: median, 504 kg; range, 450–555 kg; proportions of mares: four heaves-affected horses, and five control horses).

### Lung Function

Under conditions of low antigenic exposure, the lung function of heaves-affected horses was not different from that of control horses. Antigenic exposure induced significant airway obstruction only in diseased animals. Figure 1 depicts pulmonary resistance and elastance after 3 months of antigen avoidance (baseline) and after 1 and 30 days of exposure to indoor antigens.

### BAL

Antigenic exposure induced a significant increase in the percentage of neutrophils and mast cells in heaves-affected horses after 1 and 30 days, along with a decrease in the percentage of lymphocytes. In control horses, a transient increase in neutrophils was also present on Day 1 (Figure 2). In absolute cell counts, no significant change was evident over time in the number of total cells, lymphocytes, macrophages, or eosinophils recovered from BAL in either group. An increase from baseline occurred in neutrophils and mast cells in both groups after 1 day and in mast cells after 30 days in heaves-affected horses (Table 1).

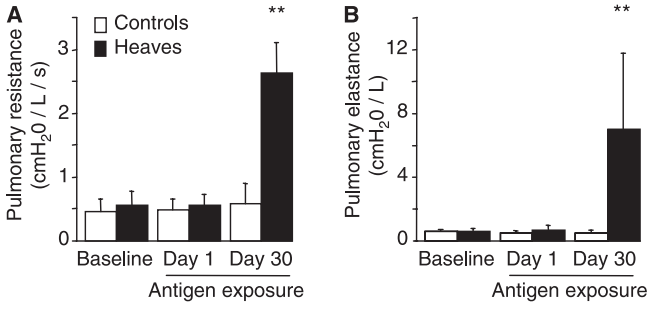


*Figure 1.* Pulmonary resistance (*A*) and elastance (*B*) after 3 months of antigen avoidance (Baseline) and after 1 and 30 days of antigenic exposure. Heaves-affected horses, $n = 6$; control horses, $n = 5$. Mean ± SD. **Different from Baseline and Day 1 within the same group, and different from controls at the same time point, $P < 0.01$.

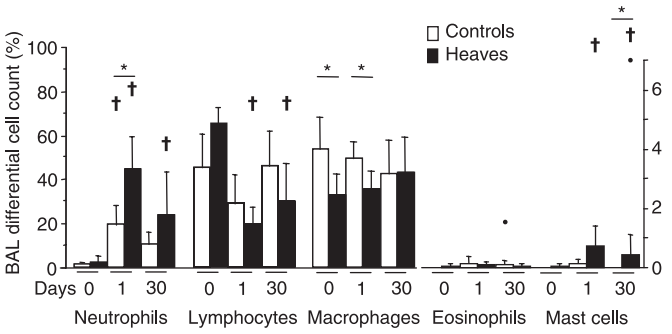


***Figure 2.*** Differential cell count of bronchoalveolar lavage (BAL) after 3 months of antigen avoidance (baseline = Day 0), and after 1 and 30 days of antigenic exposure. Heaves-affected horses, $n = 6$; control horses, $n = 5$. Mean ± SD. *Different between groups at one time point. †Different from baseline within the same group, $P < 0.05$. •Outliers.

### Airway Smooth Muscle in Peripheral Airways

Figure 3A illustrates ASM stained by immunohistochemistry in a peripheral airway. Individual airways of heaves-affected horses exhibited increased ASM mass, which was more pronounced in the most peripheral airways (Figure 4). In horses with heaves, mean ASM mass (Figure 5A) and myocyte nuclei per perimeter length (Figure 5B) were approximately 2-fold greater than in control horses, and were unaffected by a 30-day antigenic exposure. The greater ASM mass observed was not associated with a higher cell density (ASM nuclei per ASM area), but with a significantly lower density (Figure 5C). These findings suggest contributions by both hyperplasia and hypertrophy to ASM remodeling in peripheral airways, with a greater contribution of hyperplasia.

### Proliferation and Apoptosis of Airway Smooth Muscle in Peripheral Airways

At baseline, both $PCNA^+$ myocytes and $TUNEL^+$ myocytes (Figures 3C and 3D) were more numerous in heaves-affected horses than in control horses, which suggests that chronic ASM remodeling is associated with an increased cellular turnover, because both proliferation and apoptosis were increased (Figures 6A and 6B, baseline). This high turnover was unaffected by the 30-day exposure. In contrast, control animals showed an increase in both proliferative and apoptotic cells after antigenic exposure, compared with baseline ($PCNA^+$, 2.1-fold increase, $P = 0.01$; $TUNEL^+$, 2.4-fold increase, $P = 0.06$) (Figure 6). This increase remained below the level observed in heaves-affected horses, and was not associated with a change in ASM mass (Figure 5A).

### Airway Smooth Muscle Proliferation and Apoptosis in Endobronchial Biopsies

At baseline, no difference was evident between groups in terms of ASM area percentage (Figures 3B and 7A), proliferative density ($PCNA^+$ cells/ASM area) (Figure 7C), and percentage of proliferating airway myocytes (Figure 7D), or in terms of apoptotic density ($TUNEL^+$ cells/ASM area) (Figure 7E) and percentage of apoptotic myocytes (Figure 7F). Only a modest increase in myocyte density (myocyte nuclei/ASM area) was evident in heaves-affected animals (Figure 7B). After antigenic exposure, the ASM area percentage decreased in heaves-affected horses (i.e., biopsies performed during ongoing bronchospasm), but remained stable in control animals. The proliferative density and percentage of proliferating airway myocytes were significantly greater in heaves-affected horses after antigenic exposure, compared with control horses (Figures 7C and 7D). No significant difference was evident between groups and time in terms of $TUNEL^+$ myocytes (Figures 7E and 7F). Interestingly, approximately 50% of $PCNA^+$ ASM cells were found in clusters of three or more cells (data not shown).

## DISCUSSION

In this study, we examined the effects of antigenic exposure on ASM mass, proliferation, and apoptosis in the peripheral and central airways of mature animals with preexisting ASM remodeling that had been through multiple cycles of antigenic exposure, bronchospasm, and inflammation throughout their lives. After this natural challenge, only heaves-affected horses developed airway obstruction and sustained BAL inflammation. In peripheral airways, ASM remodeling appears to have reached a new dynamic equilibrium, characterized by a high cellular turnover where ASM mass, myocyte number, and markers of proliferaton and apoptosis were increased compared with control horses, but were unaffected by an antigenic challenge. Antigenic exposure increased markers of proliferation and apoptosis in control horses without affecting their ASM mass. In the central airways, proliferative myocytes were increased in diseased animals only after challenge, and antigenic exposure

**TABLE 1. CELL COUNTS IN BRONCHOALVEOLAR FLUID**

| | | Baseline | Antigenic Exposure (1 Day) | Antigenic Exposure (30 Days) |
|---|---|---|---|---|
| Total cell count ($\times 10^7$) | C | 4.17 (1.45) | 7.13 (2.69) | 7.97 (0.73) |
| | H | 3.45 (3.59) | 8.82 (6.91) | 5.91 (13.08) |
| Neutrophils ($\times 10^7$) | C | 0.04 (0.02) | 1.53 (1.16)* | 0.80 (0.43) |
| | H | 0.03 (0.06) | 4.28 (4.29)* | 0.90 (1.80) |
| Lymphocytes ($\times 10^7$) | C | 2.02 (1.16) | 1.95 (0.97) | 3.69 (1.40) |
| | H | 2.20 (2.09) | 1.48 (0.97) | 2.00 (4.50) |
| Macrophages ($\times 10^7$) | C | 2.11 (0.51) | 3.50 (1.32) | 3.36 (1.28) |
| | H | 1.21 (1.53) | 2.96 (2.00) | 3.03 (6.78) |
| Eosinophils ($\times 10^5$) | C | 0.00 (0.00) | 1.09 (1.49) | 3.42 (5.56) |
| | H | 0.09 (0.21) | 0.95 (1.58) | 0.02 (0.06) |
| Mast cells ($\times 10^5$) | C | 0.00 (0.00) | 1.08 (1.17)* | 0.00 (0.00) |
| | H | 0.01 (0.01) | 5.65 (6.61)* | 0.46 (0.73)*† |

*Definition of abbreviations*: C, control horses; H, horses with heaves.
Values are presented as mean (SD).
* Different from baseline within the same group, $P < 0.05$.
† Different between groups at one time point, $P < 0.05$.

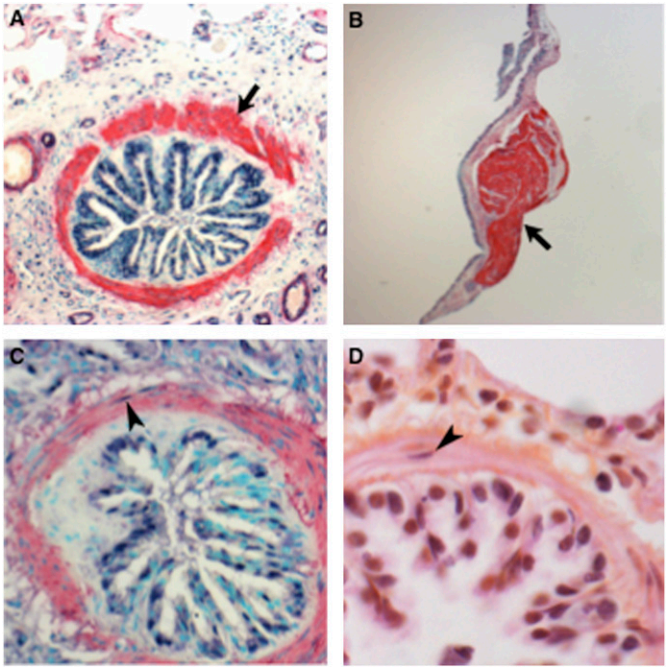


*Figure 3.* Airway smooth muscle (*arrows*) (smooth muscle α-actin stained by immunohistochemistry) in peripheral lung biopsies (*A*) and endobronchial biopsies (*B*). Airway myocytes were positive for proliferating cell nuclear antigen (PCNA) (C) and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) (*D*) (*arrowheads*). Original magnifications: ×10, ×4, ×20, and ×40 for *A*, *B*, *C*, and *D*, respectively.

exerted no measurable effect on control horses. We confirmed our hypothesis, which stated that heaves-affected horses have greater ASM mass than age-matched control horses, and that this remodeling is maintained in part by hyperplasia. However, a month-long antigenic exposure did not further increase ASM mass, and we could not correlate the changes seen in peripheral airways with the changes in endobronchial biopsies.

### Inflammation, Bronchoconstriction, and Remodeling

Exposure to indoor antigens induced marked and persistent airway neutrophilia in heaves-affected horses. The neutrophil percentage in BAL, more than in absolute cell counts, has proven useful in monitoring environment-induced airway inflammation in horses (15), but healthy animals can also develop transient inflammation under similar conditions (25). That was the case here, although to a lesser extent than in heaves-affected animals, and without the development of concomitant airflow limitation or increased ASM mass. The present study indicates that the transient inflammation resulting from antigen exposure in healthy animals also leads to an up-regulation of ASM turnover (seen in peripheral airways), without an association with airway obstruction or thickening of the ASM. The inflammation-induced up-regulation of ASM turnover therefore appears to be part of a normal response in healthy subjects. However, we cannot conclude whether ASM remodeling in diseased animals is only the result of a greater and more persistent inflammation, or is the result of factors intrinsic to ASM, as suggested by *in vitro* studies (26, 27).

### ASM Remodeling in Peripheral Airways and Lung Function

Using postmortem lung samples, Herszberg and colleagues (18) showed that horses with heaves manifest more ASM in their medium and small airways than horses without respiratory disease. In the present study, we confirmed and extended these findings by housing age-matched control horses in the same environment and by examining the effects of antigenic exposure on parameters of remodeling. Although all the heaves-affected horses had a greater mean peripheral ASM mass than did the control horses, this mass was not correlated with pulmonary resistance or elastance when horses were symptomatic (data not shown). This finding is in agreement with the findings of James and colleagues (5), in which small airway smooth muscle thickness was not correlated with severity of asthma, despite an increase in thickness compared with control samples. In that study of human asthma using postmortem specimens, only ASM in medium and large airways was associated with disease severity. This result is unlikely to mean that ASM remodeling in small airways is irrelevant, but more likely that clinical signs and criteria of severity based on need for therapy (as in James and colleagues [5]) or conventional assessments of pulmonary function correlate better with larger airway bronchospasm. Moreover, when horses were asymptomatic (at baseline), no difference in lung function was detectable, despite the presence of ASM thickening. This finding is in agreement with a mathematical model predicting that ASM thickening without concurrent bronchoconstriction exerts only a mild effect on airway caliber and lung function (28). More refined techniques to assess function, and especially peripheral airway obstruction, may have shown low-grade persistent airflow obstruction.

### ASM Remodeling in Peripheral Airways: Contribution of Hyperplasia to ASM Remodeling

Horses with heaves have more than twice as much ASM mass and approximately twice as many airway myocytes in their peripheral airways as age-matched control animals. This alteration in ASM is associated with an increase in proliferating airway myocytes, rather than a decrease in apoptosis. These results are consistent with a previous study on postmortem equine lung tissue (18). The relatively greater increase in proliferating myocytes than in ASM mass (4.2-fold versus 2.3-fold at baseline, respectively) suggests that *in situ* proliferation accounts for some of the increase in ASM, even if the percentage of proliferating airway myocytes was not signifi-

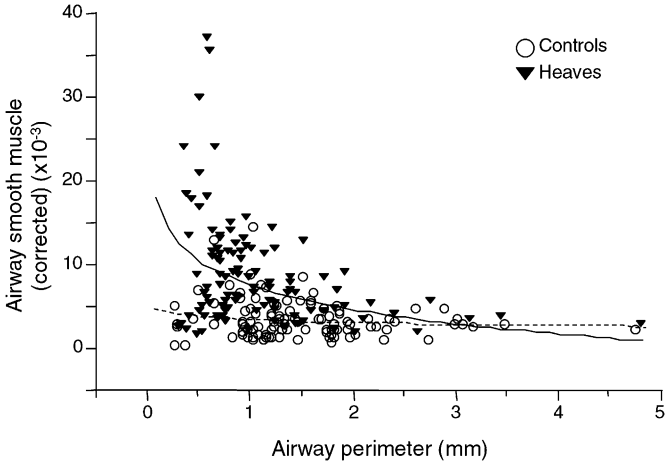


*Figure 4.* Airway smooth muscle area corrected for internal perimeter squared in peripheral airways at both time points. The internal perimeter of the airways in cross sections ranged from 0.2–4.8 mm (*x*-axis). Logarithmic regression lines illustrate trends for diseased animals (*solid line*) and control horses (*dashed line*). Control horses, $n = 108$ airways. Horses with heaves, $n = 109$ airways.

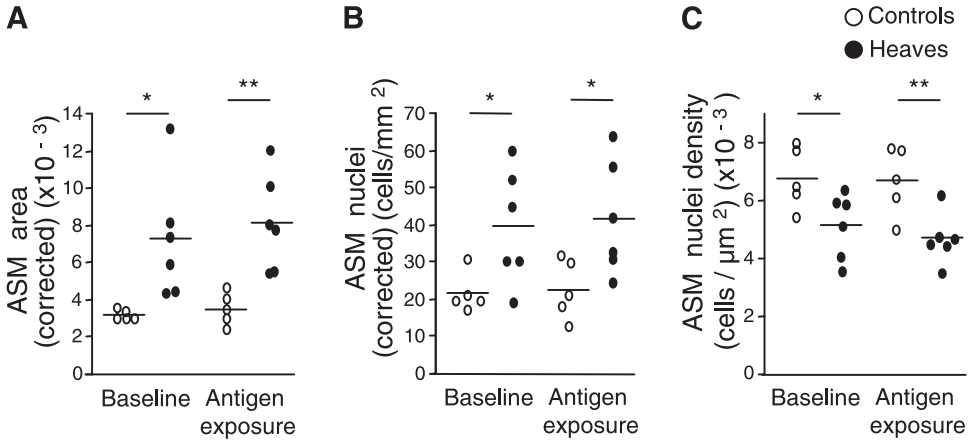


***Figure 5.*** Airway smooth muscle (ASM) area (*A*) and ASM myocyte nuclei (*B*) corrected for internal perimeter squared, and (*C*) myocyte density (ASM nuclei/measured ASM area) after 3 months of antigen avoidance (Baseline) and after 30 days of antigenic exposure. Heaves-affected horses, $n = 6$; control horses, $n = 5$. Horizontal bars represent the mean. $*P < 0.05$, $**P \leq 0.01$.

cantly different in the peripheral airways (data not shown). The differences in myocytes per airway perimeter squared and per measured surface suggest a contribution of both hyperplasia (i.e., increased mass resulting from increased cell number) and cellular hypertrophy. The contribution of cellular hypertrophy is consistent with the increased cell size described in patients with asthma (3, 9, 29), but hypertrophy was not demonstrated in all patients (7, 30). The decrease in myocyte nuclei per unit area could also be attributable to an increase in extracellular matrix deposition within the smooth muscle bundle, because we did not measure cell size directly. Nevertheless, these phenomena are not mutually exclusive, and in asthma, evidence exists of both hypertrophy and hyperplasia (3, 9, 29, 31, 32), along with increased extracellular matrix (33, 34), and with possible regional differences within the bronchial tree (3). Finally, evidence that ASM hyperplasia in chronic ASM remodeling is at least partly attributable to *in situ* proliferation suggests that limiting the proliferative capacity of airway myocytes may be of therapeutic value, even without directly inducing cell death.

### ASM Remodeling in Peripheral Airways: Limited Effect of Antigenic Exposure in Heaves-Affected Horses

The parameters of ASM remodeling were not affected by a month-long antigenic exposure in heaves-affected horses. The lack of further increase in ASM mass or cell number suggests that ASM remodeling may reach a plateau after a certain mass is attained. By showing that ASM remodeling can occur early in the natural progression of asthma (35) and that ASM thickening correlates better with severity than with duration, James and colleagues (5) findings indirectly support this concept of plateau or equilibrium in ASM remodeling. This plateau, or dynamic equilibrium, may prevent complete airway obstruction by ASM (a phenomenon not known to occur in heaves or in fatal asthma). Interestingly, antigenic exposure was associated with a modest but significant rise in proliferative and apoptotic cells only in control animals, which developed transient inflammation without airflow limitation. Taken together, these results suggest that the normal response to a transient inflammatory event is an increase in myocyte proliferation and an appropriate compensatory increase in apoptosis, whereas more pronounced or persistent inflammation leads to an increase in ASM mass in diseased animals. We postulate that after the ASM mass reaches a new dynamic equilibrium, as in the peripheral airways of chronically affected horses, myocytes show an elevated baseline turnover that is no longer affected by antigenic exposure. Ongoing tissue inflammation may also persist in asymptomatic heaves-affected horses, and may contribute to the ongoing proliferation and apoptosis observed at baseline. However, it is difficult to predict whether a longer exposure could have led to an alteration in this dynamic equilibrium in heaves-affected horses. Because the control horses have spent most of their lives exposed to hay, longer exposure would not likely have led to an increase in their ASM mass.

### ASM Remodeling in Central Airways: Bronchospasm Affects ASM Quantification in Equine Endobronchial Biopsies

At baseline, ASM area percentage in endobronchial biopsies was not significantly increased in heaves-affected horses. Most

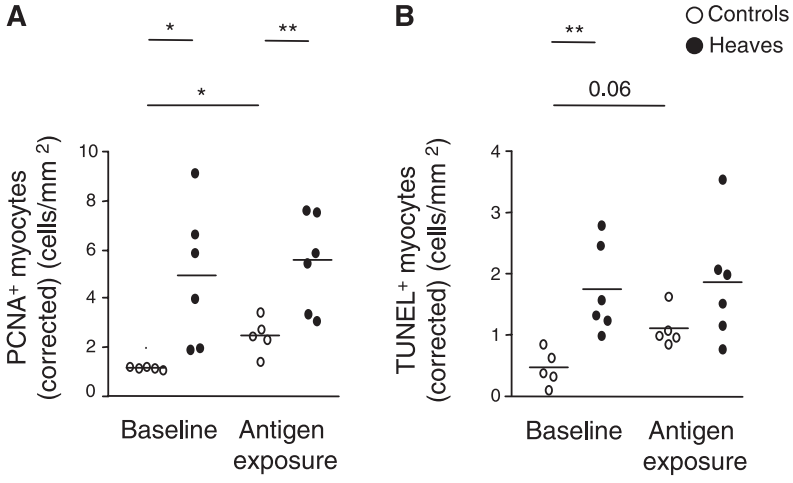


***Figure 6.*** Proliferation and apoptosis in peripheral airways. $PCNA^+$ (*A*) and $TUNEL^+$ (*B*) myocytes were corrected for internal perimeter squared after 3 months of antigen avoidance (Baseline) and after 30 days of antigenic exposure. Heaves-affected horses, $n = 6$; control horses, $n = 5$. Horizontal bars represent the mean. $*P < 0.05$, $**P \leq 0.01$.

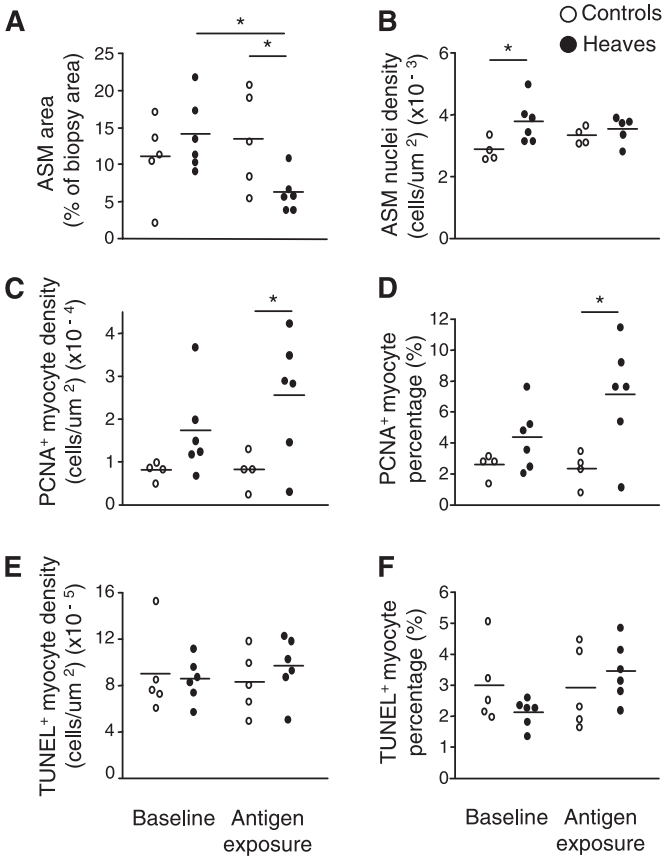


***Figure 7.*** Airway smooth muscle in endobronchial biopsies. (*A*) Percentage of ASM area (ASM area/biopsy area). (*B*) Myocyte density (myocyte nuclei/ASM area). (*C*) PCNA$^+$ myocyte density (PCNA$^+$ myocytes/ASM area). (*D*) Percentage of PCNA$^+$ airway myocytes. (*E*) TUNEL$^+$ myocytes density (TUNEL$^+$ myocytes/ASM area). (*F*) Percentage of TUNEL$^+$ airway myocytes. Biopsies were performed after 3 months of antigen avoidance (Baseline) and after 30 days of antigenic exposure. Heaves-affected horses, $n = 6$ (except for *B*, exposure $n = 5$); control horses, $n = 5$ (except for *B–D*, $n = 4$). Horizontal bars represent the mean. *$P < 0.05$.

studies of human patients found an increase in ASM area (total or percentage) (6, 8, 9, 35), although a few could not demonstrate a difference between groups (36), or else the difference was evident only in patients with severe but not intermittent asthma (9). In the present study, a significant decrease in ASM area percentage was observed after antigenic exposure. Kelly and colleagues (37) noted a similar decrease 24 hours after an allergen challenge in patients with asthma, which they attributed to a dedifferentiation to myofibroblasts. The decrease of ASM ratio after exposure could indeed represent an increase in other subepithelial components, but we also found that sampling during ongoing bronchospasm made the positioning of the biopsy forceps more difficult and the sampling more likely to be superficial, because the carinae thicken with constriction. Alternatively, the lengthening of the airways that may occur during hyperinflation (38) could increase the distance of the ASM from the carinae. Furthermore, two-dimensional morphometric analysis may not be appropriate for the quantification of equine ASM using endobronchial biopsies, regardless of clinical stage, because of the smaller size of the forceps relative to the bronchial carina. Because these results suggest that bronchoconstriction may affect morphometric measurements, a bronchodilator could be used at the time of sampling to avoid this possible confounding factor in the future.

### ASM Remodeling in Central Airways: Evidence of ASM Cell Hyperplasia in Endobronchial Biopsies

Despite the difficulties in quantifying ASM in endobronchial biopsies, data on proliferating and apoptotic myocytes were still obtained. The greater proliferation density and percentage of proliferating airway myocytes in heaves-affected horses after antigenic exposure, without a concurrent increase in apoptosis, are suggestive of *in situ* proliferation, even if no increase in ASM mass was demonstrated in these biopsies. The observation that approximately 50% of proliferating myocytes were found in clusters also supports the presence of *in situ* proliferation, possibly by the autocrine feed-forward mechanism described by Johnson and colleagues (39), or because of a localized source of growth factors. ASM proliferation was only recently shown to play a role in ASM remodeling in patients with severe, long-standing asthma (40). Previously, James and colleagues (41) found a percentage of PCNA$^+$ myocytes in a range similar to the one detected here (5–8%), but with no difference between patients with asthma and control subjects, whereas others failed to detect proliferation markers (PCNA, Ki67, or cyclin D1) in ASM bundles (9, 42, 43). However, in our study, the difference was significant only after antigenic exposure, and after subjects became symptomatic and had gone untreated for a prolonged period, which is less likely to occur in patients with asthma. The severity of disease (7) or the use of corticosteroids (42) can also account for some variations, but the processes leading to ASM hyperplasia may also differ among species (44).

This equine model of chronic asthma produced evidence of hyperplasia associated with *in situ* proliferation, as well as of possible hypertrophy in remodeled ASM. An antigenic exposure had no effect on morphometric measurements in peripheral airways and little effect on the percentage of proliferating myocytes in endobronchial biopsies in these chronically affected animals. These findings are in agreement with the concept that ASM remodeling is an early event in asthma and remains stable in terms of mass later in life (5, 45). We conclude that in peripheral airways at least, ASM remodeling reaches a new dynamic equilibrium in which the increased mass is maintained with an elevated turnover. This finding also suggests that limiting the proliferative capacities of airway myocytes may have a therapeutic value, even without directly inducing cell death.

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ENSEIGNEMENT

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PUBLICATIONS

Articles

1. Lavoie-Lamoureux A, Leclere M, Lemos K, Wagner B, Lavoie JP. Markers of systemic inflammation in Horses with Heaves. *J Vet Intern Med*. 2012. doi: 10.1111/j.1939-1676.2012.00993.x. Epub ahead of print.
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11. Leclere M, Lavoie JP, Dunn M, Bédard C. Evaluation of a modified thrombelastography assay initiated with recombinant human tissue factor in clinically healthy horses. *Vet Clin Pathol*. 2009 Dec; 38(4):462-6.
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Conférencière invitée

1. Leclere M. Sous la loupe du Réseau Équin: AIE, EEE et autres maladies sous surveillance. Association des médecins vétérinaires praticiens du Québec. Boucherville, Canada. 2011.
2. Leclere M. Dexaméthasone, Prednisone, Prednisolone : du pareil au même? Association des médecins vétérinaires praticiens du Québec. Saint-Hyacinthe, Canada. 2009.
3. Leclere M, Lavoie-Lamoureux A, Martin JG, Lavoie JP. Effect of antigen challenge and withdrawal on airway smooth muscle remodeling in heaves. Research report conference. American College of Veterinary Internal Medicine Convention. Montréal, Canada. 2009.
4. Leclere M. Equine Influenza. Conférencière invitée par l'Ontario Association of Equine Practitioners (éducation continue). Guelph, Canada. 2007.
5. Leclere M. Urgences médicales du poulain nouveau-né. Conférencière invitée par l'Association des médecins vétérinaires praticiens du Québec (éducation continue). Saint-Hyacinthe, Canada. 2006.

Communications scientifiques (présentatrice lorsque le nom est souligné)

1. Lavoie-Lamoureux A, Leclere M, Lemos KR, Lefebvre J, Wagner B, Lavoie, JP. Systemic Inflammation is Present in both Remission and Clinical Exacerbation in an Equine Model of Severe Asthma. Poster presentation. American Thoracic Society International Conference. Denver, CO, USA. 2011.
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